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Production and effects of IL-6 on human osteoblast-like cells

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Production and Effects of IL-6 on Human Osteoblast-like cells.

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This thesis is dedicated to my Mum, Dad and Dean
who's help and guidance have been invaluable to me

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SUMMARY

There is increasing evidence that IL-6 may function as a bone-active cytokine. IL-1, which itself exerts potent effects on bone cells, is a potent stimulator of IL-6 release in several connective tissue cell types. Furthermore, these cytokines exhibit strikingly similar biological activities in other tissues. It is therefore possible that IL-6 may mediate the effects of IL-1 in bone. In this thesis, the synthesis and factors regulating the production of IL-6 protein and mRNA by human osteoblast-like cells was investigated. In addition, the effects of IL-6 on human osteoblast-like cell function was determined. A rat osteosarcoma cell line was used as a comparative osteoblast-like model.

The cytokines IL-1 and TNF, but not the osteotropic hormones PTH and $1,25(OH)_2D_3$, induced the synthesis of IL-6 mRNA and protein from human osteoblast-like cells whereas PTH was the most potent stimulator of IL-6 protein release in the rat osteosarcoma cells. Despite the high levels of production of IL-6, this cytokine exerted no effect on various osteoblast cell activities including cell proliferation, cytokine and prostaglandin release and expression of differentiated phenotype.

Although human osteoblast-like cells apparently lacked responsiveness to IL-6, Northern and dot blot analysis of IL-6 receptor mRNA showed that the transcript was constitutively expressed but not modulated by any of the factors above. Lack of effects of IL-6 on osteoblasts could be due to the expression of non-functional receptors.

To provide a clearer understanding of the possible role of IL-6 in bone physiology *in vivo*, IL-6 mRNA production in actively remodelling human bone tissue was studied by *in situ* hybridisation. Expression of this cytokine was observed in many cell types including cells with the appearance of newly formed osteoblasts as well as osteoblasts and osteoclasts adjacent to the bone surface and marrow stromal cells. The demonstration of the presence of IL-6

in bone sections shows that IL-6 production by osteoblasts in culture is not artifactual. Further *in situ* hybridisation studies are required to co-localise IL-6 mRNA with osteoblast and osteoclast markers in order to gain more information about the role of this cytokine *in vivo*. That several cell types are apparently capable of synthesising IL-6 in the bone microenvironment provides strong evidence that IL-6 may co-ordinate certain cellular events within bone such as hematopoiesis. However, the failure of IL-6 to affect osteoblast activity *in vitro* may discount a direct role in bone formation. The importance of osteoblast-derived IL-6 and its involvement in normal bone remodelling remains to be elucidated.

CHAPTER 1

INTRODUCTION

Bone function

Apart from the obvious structural and supporting properties, bone has a number of other important functions. Bone provides an attachment site for tendons and ligaments and is protective for internal body organs such as the heart and central nervous system. Bone also serves as a reservoir for several mineral ions such as calcium, phosphorus and magnesium which are capable of being released into the circulation during the process of bone remodelling. Bone therefore plays an active part in mineral homeostasis.

Bone structure

There are three types of bone, cortical, trabecular (or cancellous) and woven bone.

Cortical bone

Cortical bone constitutes approximately 80% of the total bone mass. It lines the external surfaces of all bones and is most abundant in shafts of long bones where trabecular bone is sparse. Cortical bone is composed of Haversian systems (or osteons) containing osteogenic cells and canals formed by the remodelling cycle and through which blood vessels run. Haversian systems form a tightly packed, longitudinally aligned conformation surrounded by a cement line that canaliculi are unable to penetrate. The outer and inner most layers of the cortical bone have no Haversian systems but instead contain lamellae deposited in parallel to the periosteal and endosteal surfaces.

Cortical bone mainly exerts a structural and protective role. As it is only slowly remodelled, it does not play a major role in mineral homeostasis.

Whether cortical and trabecular bone communicate with each other has yet to be determined.

Trabecular bone

Trabecular bone, with its lattice-like structure, comprises 20% of the skeleton and is found predominantly in the long bones and the vertebrae.

Trabecular bone contains a more loosely arranged Haversian system particularly because of the high degree of porosity in trabecular bone. Instead, the trabeculae consist of a series of perforated interconnecting plates of bone surrounded by blood vessels, hematopoietic marrow and fat. This type of bone provides mechanical strength as orientation of trabeculae reflect the pattern of stress that a particular area of bone is subject to.

Trabecular bone is highly cellular and so it is associated with all cells involved in the remodelling cycle. It is therefore more metabolically active than cortical bone and possesses a higher bone turnover rate. As this bone is remodelled more frequently, its role in mineral homeostasis is of major importance.

Woven bone

This immature bone represents the initial bone laid down in the foetus and around sites of active fracture repair. It is not structurally organized and the collagen fibrils are layed down in an irregular random fashion. Woven bone is temporary and is eventually remodelled to form either cortical or trabecular bone.

Bone histiogenesis

Bone is derived from the mesoderm during embryonic development. There are two types of bone formation; intramembranous ossification for flat bones and endochondral ossification for long bones, the difference being the presence or

absence of a cartilagenous template.

Intramembranous ossification

During intramembranous ossification, a group of mesenchymal cells within the connective tissue divide and differentiate into osteoblast precursors and mature osteoblasts. Osteoblasts then deposit immature woven bone with its disordered collagen fibrils. Furthermore, calcification does not proceed in an orderly fashion. The bone becomes invaded with blood vessels and hematopoietic bone marrow and is eventually remodelled to form mature lamellar bone.

Endochondral ossification

Mesenchymal connective tissue differentiates into prechondroblasts and chondroblasts. Once these cells have secreted the cartilagenous matrix and become embedded, they are known as chondrocytes. Unlike osteocytes (embedded in trabecular bone and formed from osteoblasts), chondrocytes retain the capacity to divide. New matrix is synthesized between the chondrocytes referred to as interstitial growth and the chondrocytes are arranged as regular columns within the growth plate. This is known as the proliferation zone and together with differentiation, is responsible for the increased length of tubular bone. The chondrocytes enlarge in the hypertrophic zone and progressively lose their intracytoplasmic glycogen and show increased vacuolation. This prepares the matrix for calcification. The hypertrophic chondrocytes die in the region of matrix that selectively calcifies. Once calcified, the matrix is partially resorbed by osteoclasts and becomes invaded by blood vessels in a region termed the metaphysis. Osteoblasts form woven bone over the cartilagenous remnants. The trabeculae formed are further remodelled lower in the growth plate to attain the mature state known as secondary spongiosa.

Cells of bone

Bone turnover in the adult skeleton is continually occurring at discrete sites along the bone surface. The cells responsible for dissolution of matrix are known as osteoclasts and represent a completely different lineage and phenotype from the bone forming cells, the osteoblasts. The necessity for remodelling is evident during fracture repair thereby 'knitting' the bones together. Other reasons are mineral homeostasis, alterations in mechanical stresses and replacement of old bone for new.

Osteoclasts

Lineage

Osteoclasts are derived from hematopoietic mononuclear cells in bone marrow (Roodman et al 1985). Mononuclear osteoclast precursors can circulate in the blood and proliferate and fuse at endosteal surfaces to produce multinucleated osteoclasts. The majority of evidence supports the view that the precursors are derived from a pluripotent stem cell able to differentiate into a granulocyte, monocyte or osteoclast. The most likely progenitor is therefore CFU-GM (colony forming unit-granulocyte/macrophage). Osteoclasts are not derived from more mature monocytes and macrophages (Vaes 1988, Chambers 1989). For a review of this subject refer to Skjodt and Russell (1991).

Structure

Osteoclasts are large multinucleated cells of approximately 50-100 um diameter and are located on endosteal bone surfaces associated with calcified bone matrix at the interface with bone marrow (Baron 1989). In normal adult bone, osteoclasts are not frequently detected but are more readily found at sites of bone undergoing active

remodelling. Under these circumstances, osteoclasts can contain up to 100 nuclei, although 10-20 nuclei of heterogeneous size and shape are evident under more normal conditions. Osteoclasts also possess a strongly basophilic cytoplasm containing primary lysosomes, numerous mitochondria (Holtrop and King 1977) and golgi complexes (Baron et al 1985). One of the most striking features of the osteoclast is the possession of a highly folded membranous area known as the ruffled border, which is in intimate contact with the bone surface (reviewed by Jones et al 1985). The ruffled border is surrounded by a clear zone which is free of organelles and contains actin filaments which anchor and seal the ruffled border to the bone surface (Holtrop and King 1977). Osteoclasts are motile cells and so once they have completed the resorption of a pit, are capable of subsequent resorption activities (Ali et al 1984).

Function

Osteoclasts are capable of solubilizing the mineral content of matrix and causing the dissolution of organic matrix components (Ali et al 1984, Vaes 1988). The area under the ruffled border that is enclosed by the clear zones, is responsible for the resorption of bone. Lysozomal enzymes are synthesized by the osteoclast and are found in the endoplasmic reticulum, golgi and secretory vesicles. The enzymes are secreted across the ruffled border into the extracellular bone resorbing compartment (Gay and Mueller 1974). Hydrogen ions are formed within the cell by the enzyme carbonic anhydrase (isoenzyme II). Osteoclasts also possess a proton pump (Baron et al 1985) which is capable of exporting the hydrogen ions into the resorbing compartment thereby creating an acidic environment which is optimal for the proteolytic enzymes (Vaes 1968). Tartrate-resistant acid phosphatase (TRAP) (Minkin 1982) and lysosomal enzymes such as cathepsins (Delaisse et al 1984) are produced by osteoclasts. Delaisse et al (1984) observed that excretion of lysosomal

cysteine proteases by PTH-stimulated embryonic mouse calvariae in culture correlated with the extent of resorption which was evaluated by increased serum hydroxyproline and calcium levels. Osteoclasts are capable of degrading the bone matrix extracellularly (Baron et al 1985) and it appears that the mannose 6-phosphate receptor is involved in the directional release of the lysosomal enzymes (Baron et al 1988).

Although TRAP activity may not be exclusive to osteoclasts, its degree of expression is certainly far higher in osteoclasts associated with the bone surface (Dodds and Gowen 1991). Since TRAP is a hydrolytic enzyme which may be involved in the dissolution of matrix, these findings provide good evidence that TRAP may be active during resorption.

Osteoblasts

Lineage

Using diffusion chambers implanted in vivo, stromal tissue within bone marrow rather than hematopoietic tissue was found to be the origin of the osteoblast (reviewed by Owen 1985). It is likely that a pluripotent stem cell within stromal marrow can give rise to osteoblasts, fibroblasts, chondroblasts and other stromal cells of the marrow cavity (Owen 1985).

Tritiated thymidine pulse treatment in rabbits has been used to follow the development of cells throughout the osteogenic lineage (Kember 1960, Owen 1963). Radiolabelled nuclei were first detected in preosteoblasts within 30 minutes. followed by a lag period before labelling in osteoblasts and later in osteocytes were observed.

Preosteoblasts

These are committed progenitor cells capable of proliferation and expressing low levels of alkaline phosphatase enzyme. They are usually present close to where active mature osteoblasts are synthesizing bone and the preosteoblasts become more mature as they reach the bone surface (Puzas 1990).

Osteoblasts

Osteoblasts do not appear to function individually but as members within a team, where they are seen as clusters of plump cuboidal cells along the bone surface lining a layer of osteoid.

Osteocytes

Approximately 10-20% of the osteoblasts eventually become encased in calcified bone (Menton et al 1984). The cells are then known as osteocytes. They are metabolically less active than osteoblasts but possess processes, which may form a communication and nutrient system (Menton et al 1984).

Lining cells

These cells line the majority of surfaces on mature bone. They are flattened and contain few organelles unlike osteoblasts (Jones et al 1985). It is possible that lining cells can transform into osteoblasts although substantial evidence is still required (Parfitt 1987).

Osteoblast structure

Osteoblasts are mononuclear cells, 20-30 um in diameter, and contain polarized intracellular organelles. These include an extensive network of rough endoplasmic reticulum containing large amounts of ribosomes associated with the high synthetic

activity of osteoblasts. They also contain an intricate golgi apparatus and other intracellular components reflecting the high metabolic activity of these cells. Osteoblasts are capable of extending cytoplasmic processes and may therefore be able to communicate with other cells within the bone microenvironment (Vesely et al 1992). The osteoblasts are also in contact with the stomal cells of the marrow cavity (Deldar et al 1985).

Osteoblasts synthesize substantial levels of alkaline phosphatase which is located in the plasma membrane. The function of this enzyme is unclear but may be associated with bone formation. For a review on osteoblast phenotype refer to Rodan and Rodan (1983).

Function

Osteoblasts lay down matrix in resorption lacunae previously formed by osteoclastic activity. Initially the matrix is not calcified and is known as osteoid. However after a lag period, the osteoid becomes mineralised by the activity of the osteoblasts.

Matrix constituents

Collagen

Within bone 80-90% of the total bone matrix protein is comprised of type I collagen. The collagen fibrils are extensively cross linked to form a highly insoluble complex (Urist et al 1983). The type I collagen molecule consists of a triple helix containing two identical α1(I) chains and an α2(I) chain. These are tightly supercoiled because of the presence of a glycine at every third residue. In the matrix, individual collagen fibrils are packed in an organized conformation with

small spaces located between the ends of the molecules and the mineral phase is initially deposited within and subsequently between the collagen fibrils. Collagen type I is produced by the osteoblasts (Canalis 1983a) and it undergoes several post-translational modifications during its biosynthesis and secretion. Amino and carboxy terminal peptidases cleave the procollagen molecules to form the mature protein. In normal mature bone, the collagen fibrils are arranged in parallel, whereas in woven bone the fibrils are randomly deposited. The developing collagen fibril becomes the focus for the association of non-collagenous bone proteins. Several of these proteins are also produced by osteoblasts.

Osteocalcin

This bone matrix protein is also known as bone gla protein (BGP) because of the three vitamin K-dependent amino acids, γ-carboxyglutamic acid, (gla). Osteocalcin is a small (6 kD), highly conserved, abundant bone matrix protein. Osteocalcin is produced by osteoblasts from several species including human (Beresford et al 1984b). Its biosynthesis is dependant on vitamin K for gla formation from three glutamic acid residues, vitamin C for hydroxylation of the proline residues and vitamin D for the stimulation of synthesis. It exerts a weak association with calcium (Hauschka et al 1982) but has strong affinity for hydroxyapatite via the gla residues which are vital for mineral binding (Poser and Price 1979). The physiological function of osteocalcin is unknown. Long-term warfarin treatment in rats can induce the abnormal mineralization of the growth plate and premature epiphyseal closure (Price et al 1982). In humans however, vitamin K depletion has not been shown to be associated with adverse bone alterations (Piro et al 1982). Osteocalcin has been shown to have a role in bone resorption since it has been proposed to function as a chemotactic factor for osteoclast precursors (Mundy and Poser 1983). In addition, bone implants from warfarin-treated rats are poorly

resorbed *in vivo* when compared to normal bone (Lian et al 1984).

Matrix gla protein

Matrix gla protein (MGP) is a second gla-containing protein in bone and is slightly larger than osteocalcin having a molecular weight of 10 kD. MGP contains 5 gla residues (Price and Williamson 1985) and possesses some degree of homology with osteocalcin which would indicate a possible common ancestor. MGP is expressed at early stages of bone formation and precedes the accumulation of osteocalcin (Fraser et al 1988). Unlike osteocalcin, MGP is not bone specific as it is also found in cartilage. Fraser et al (1988) detected either MGP or osteocalcin in various clonal osteoblast lines which could indicate that osteoblasts are expressing these proteins at different stages of development.

Osteonectin

Osteonectin is the most abundant non-collagenous bone matrix protein which is an acidic phosphorylated glycoprotein of 32 kD molecular weight. Osteonectin possesses high affinity for binding ionic calcium and hydroxyapatite (Termine et al 1981). Its binding domains for collagen and mineral are separate and this may be indicative of a role for osteonectin in the promotion of mineral deposition between type I collagen fibrils. Osteonectin is synthesized by a number of different cell types including osteoblasts and is produced by a number of species including human (Gehron-Robey and Termine 1985). Osteonectin is associated with rapidly growing tissues including non bone tissue (Holland et al 1987) and therefore may exert a proliferative role on osteoblasts for example, as well as exerting effects on mineralization.

Osteopontin

This is a 44 kD bone phosphoprotein which may be involved in the promotion of attachment and spreading of osteoblasts, via an arg-gly-asp amino acid sequence, to the bone surface (Oldberg et al 1986). Osteopontin contains nine aspartic residues which may confer hydroxyapatite binding (Oldberg et al 1986). Previously osteopontin has been immunolocalized on osteoblasts and their precursors, however recently it has been demonstrated by *in situ* hybridization to be expressed by osteoclasts as well as osteoblasts (Littlewood et al 1991a, Merry et al 1992b). Heinegard and Oldberg (1989) demonstrated the presence of osteopontin at the clear zone of the osteoclast binding to the mineral and postulated that osteopontin, also present in the matrix, may be involved in the attachment of the osteoclasts to the bone surface via the vitronectin receptor.

Sialoproteins

Bone sialoprotein was so called because of its high sialic acid content (20% by weight). It is a 23 kD protein and is considered to be bone specific. It is not located in any other tissue *in vivo* except for low levels in dentine (Fisher et al 1983). Bone sialoprotein is produced by osteoblast-like cells (Heinegard and Oldberg 1989) yet its function is unknown although it binds calcium and so may be involved in the bone mineralization process. In other tissues, similar highly acidic proteins have been shown to affect cell adhesion (Edelman 1983) and so bone sialoprotein may have a similar function. Also, like osteopontin, it contains the arg-gly-asp sequence (Oldberg et al 1986) which enables attachment to the vitronectin receptor which is expressed by osteoclasts (Horton 1990).

Proteoglycans

These are macromolecules with acidic polysaccharide side chains known as glycosaminoglycans (GAG) attached to a central protein core. The two types of GAG found in bone are chondroitin sulphate and heparan sulphate. The latter may be involved in the interaction of osteoblasts with the extracellular macromolecules (Beresford et al 1987). They possess a negative charge and are able to bind several growth factors, eg heparan sulphate is capable of binding fibroblast growth factor (FGF). Furthermore, in order for FGF to bind to its receptor, prior association of FGF with heparan sulphate is required (Ruoslahti and Yamaguchi 1991). Most GAGs are attached to two small proteoglycan core proteins in bone, namely biglycan and decorin. Biglycan is especially abundant in developing bone and is capable of binding two GAG molecules whereas decorin is found throughout the many stages of bone development and only binds one GAG. Decorin is capable of binding to collagen fibrils and may control the rate of formation and the size of the collagen fibrils. Decorin is capable of binding Transforming growth factor beta (TGFβ) (discussed on page 31) which can neutralize the growth factor activity. In addition, TGFβ induces the synthesis of decorin and so this may represent a negative feedback mechanism for controlling TGFβ activity (Ruoslahti and Yamaguchi 1991). As certain proteoglycans are capable of binding various factors, the extracellular matrix may act as a store for growth factors. Growth factors act on target cells over a short range only and so the immobilization at the cell surface or in the extracellular matrix through binding to proteoglycans, may achieve this goal. Beresford et al (1987) demonstrated that human osteoblast-like cells produced proteoglycans. For a review of proteoglycans, refer to Ruoslahti and Yamaguchi (1991).

Other non collagenous bone matrix proteins

Several other proteins have also been isolated such as thrombospondin which again contains the arg-gly-asp sequence (Gehron-Robey et al 1989).

Osteoid also contains a number of plasma proteins including α2 HS glycoprotein and albumin. Their function is as yet unknown. For a review refer to Triffitt (1987).

Growth factors

Osteoblasts deposit a number of proteins in the bone such as TGFβ, bone morphogenetic proteins (BMPs), insulin-like growth factor I (IGF-I) and FGFs. For a review, refer to Canalis et al (1989). These factors are associated with osteoblast function and proliferation as well as exerting effects on osteoclasts. They are discussed in more detail later in this chapter.

Mineralization

The osteoid therefore contains tightly packed collagen fibrils complexed with a number of non collagenous proteins. The principal mineral constituent is hydroxyapatite. Other minerals such as magnesium ions are associated with extracellular matrix. The process of mineralization is still a matter of contention. Bone cells are probably involved in this process. Alkaline phosphatase, produced by osteoblasts, may promote mineralization by reducing levels of free pyrophosphate which is an inhibitor of hydroxyapatite formation (Fleisch and Bisaz 1962). Matrix vesicles are likely to be involved in initial calcification (Anderson 1976). Matrix vesicles are not normally evident in the upper proliferating zone of the growth plate where cells are actively dividing but are observed in clusters in the lower proliferative region. Vesicles associated with the hypertrophic zone contain needle-like hydroxyapatite crystals. These crystals

eventually disrupt the vesicles and grow to form nodules of hydroxyapatite which are deposited between the collagen fibrils. Several factors may control this process. For a review refer to Anderson (1976).

The remodelling cycle

The processes of bone resorption and bone formation are linked or 'coupled' in normal bone turnover in what is termed the bone remodelling cycle (Fig. 1.1). The bone remodelling cycle was first described by Frost (1964) who observed that remodelling occurred at discrete sites along the bone surface. Osteoclasts resorbed a quantity of the bone and this was then replaced with new matrix by the osteoblasts. These events were more closely studied during tooth egression in rats (Tran Van et al 1982).

Activation is initiated by the presence of mononuclear cells around the site of bone that is to be resorbed. These cells contain enzymes characteristic of the mononuclear phagocytic lineage eg. non-specific esterase. Flattened lining cells, possibly derived from osteoblasts, then may retract their cytoplasm to expose the matrix underneath and to which the mononuclear cells extend cytoplasmic processes to establish contact (Jones and Boyde 1976). These mononuclear cells start to fuse to become multinucleated osteoclasts and form ruffled borders against the bone surface. During the resorption phase, these cells erode a quantum of bone of characteristic size and depth, known as a resorption lacuna. This phase is only short lived, approximately 2 days after which the osteoclasts are replaced by large TRAP positive mononuclear cells of unknown origin. This initiates the reversal phase, a period of about 4 days, during which these cells lay down a cement line rich in glycoproteins and GAGs. Osteoblasts then attach to the cement line and there follows a much longer formation phase whereby osteoblasts lay down osteoid which is then eventually mineralized. It is possible that the osteoblasts then

transform to the flattened morphology of lining cells.

These cyclical events observed in remodelling must be tightly controlled both through systemic factors, and as discrete remodelling foci are present, some local signalling between the cells and matrix of bone must also exist.

Bone cell coupling

The term coupling essentially describes the cellular linkage between bone resorption and formation processes that occur at each resorption site. Successful coupling results in the replacement of the resorbed area with new bone associated with osteoblast activity. Although the mechanisms associated with this process are unknown, there are many ways in which coupling can proceed.

To account for the coupling phenomenon, Howard et al (1981) proposed that an osteoblast-stimulating factor is released during the process of osteoclastic bone resorption and that the stimulation of osteoblast activity leads to new bone formation. The source and identity of these local osteotropic factors is at present unknown. It is possible that they are released from the actively resorbing osteoclast or related cells. Alternatively, these factors could be incorporated into the bone matrix during bone formation and subsequently released during the phase of osteoclast resorption. The products of resorbed bone have been reported to act as chemotactic agents for the osteoblast lineage. Local factors such as TGFβ, released from the matrix during resorption, may mediate this chemotactic effect (Pfeilschifter et al 1990b). Similar functions may also apply to additional growth factors and cytokines that are active on bone. An alternative theory is that the factor which stimulates resorption also acts directly, although more slowly, on the osteoblast to cause their activation and subsequent new bone formation. Parathyroid hormone (PTH) is an example of a factor that stimulates both bone resorption and formation *in vivo* (Tam et al 1982).

Factors which influence the remodelling process

Systemic hormones

Parathyroid hormone (PTH)

PTH regulates the levels of calcium and phosphate in the blood by acting on bone and kidney. When calcium levels are low PTH stimulates the release of calcium from bone and increases reabsorption of calcium from the glomerular filtrate. PTH can also activate the 1α hydroxylase enzyme in the kidney to stimulate the production of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) which can then increase the intestinal absorption of calcium. Blood calcium can also influence PTH levels since an increase in calcium level decreases the secretion of PTH. All these events serve to maintain a constant serum calcium level irrespective of the contribution of the diet, bone metabolism and fluctuation in renal function. PTH is involved in the stimulation of bone resorption, thereby releasing calcium into the blood. This can be demonstrated in a number of bone organ culture systems including fetal rat long bones (Raisz 1963, Raisz 1965, Mundy et al 1976, Lorenzo et al 1983) and neonatal mouse calvariae (Feldman et al 1980). PTH is capable of synergising with a number of agents such as interleukin 1 (IL-1) (Dewhirst et al 1987, Sato et al 1989) and vitamin A (Raisz 1965) to further increase bone resorption. Several groups have observed that the action of PTH results in an increase in osteoclast number in these systems (Raisz 1963, Feldman et al 1980). Lorenzo et al (1983) reported that PTH-induced resorption occurred independently of the replication of osteoclast precursors, which would indicate that PTH exerts its effects through the fusion of pre-formed precursors. Teti et al (1988) observed that PTH did not induce the fusion of blood monocytes with osteoclasts concluding that the precursors may be committed in the bone marrow. Many groups have investigated

the effect of PTH in human, feline and primate long term marrow cultures. The cells within these cultures are capable of being induced by PTH to form multinucleated cells which possess several osteoclast characteristics such as TRAP activity, reactivity with a monoclonal antibody 23C6, calcitonin receptors and production of resorption lacunae on bone or sperm whale dentine (Ibbotson et al 1984, Roodman et al 1985, MacDonald et al 1987, Takahashi et al 1989). In a contrasting study, Kurihara et al (1991) demonstrated that PTH stimulated the proliferation of early osteoclast precursors rather than promoting the fusion of late precursors.

PTH is capable of stimulating already formed osteoclasts to resorb the medullary bone of Japanese quail (Miller 1978). Miller (1978) demonstrated that after 20 minutes infusion, PTH induced ruffled border and clear zone formation on inactive osteoclasts which was subsequently followed by increased plasma calcium levels. PTH stimulates carbonic anhydrase activity in mouse calvarial osteoclasts (Anderson et al 1985) and increases acid phosphatase and hyaluronate synthesis (Wong et al 1977), another indication that PTH activates pre-formed osteoclasts.

A possible mechanism by which PTH can mediate its bone resorptive effect is by stimulating the production of latent collagenase as visualised in mouse calvarial osteoblasts (Heath et al 1984), rat calvarial osteoblasts (Shen et al 1988) and UMR106-01 cells (Partridge et al 1987). Plasminogen activator, an enzyme capable of activating collagenase via an enzyme cascade, is also released by rodent (Hamilton et al 1984, Partridge et al 1987) and human (Evans et al 1990b) osteoblasts. Activated collagenase may degrade the osteoid layer thereby exposing the mineralized bone matrix for resorption by osteoclasts. Osteoblasts are capable of producing a variety of inhibitors for these enzymes indicating the involvement of a complex regulatory control mechanism in this potential process.

PTH is capable of inducing shape changes in bone cells (Lomri and Marie 1988)

and Rodan and Martin (1981) proposed that shape changes in osteoblasts could cause a mild retraction of cells leading to the exposure of the underlying matrix to the osteoclasts.

A related peptide (rp) called PTHrP is also capable of inducing bone resorption in organ culture (Raisz et al 1990). Bone resorptive effects of PTHrP are also observed *in vivo* when nude mice are transplanted with human esophageal carcinoma cells which produced IL-1α and PTHrP (Sato et al 1989). These mice developed hypercalcemia. Separate experiments were performed in order to assess the relative contribution of each agent. Both IL-1α and PTHrP increased ^{45}Ca release from prelabelled forearm bones of fetal mice but only PTHrP consistently increased serum calcium levels. Sato et al (1989) postulated that the effects of PTHrP on hypercalcemia were due to a combination of the stimulation of both bone resorption and calcium readsorption in the kidney (Sato et al 1989). Murrills et al (1990) observed that osteoclasts disaggregated from neonatal rat bone were stimulated to resorb devitalized bone with both PTH and PTHrP which resulted in an increased number of pits, although mean area of pit excavated was decreased.

Studies *in vivo* have demonstrated that long-term treatment with PTH is anabolic. Trabecular bone calcium is increased after daily sub-cutaneous injection of PTH for twelve days into rats (Hey et al 1988). Tam et al (1982) injected thyroparathyroidectomized rats with PTH and observed that the daily injection increased bone apposition while continuous infusion resulted in increased bone formation and resorption. In rats injected daily with PTH, the amount of cancellous bone eventually increased and this was accompanied by an increased number of osteoblasts and a decreased osteoclast number (Liu and Kalu 1990). Hock et al (1989) used a combination of pulsatile PTH with calcitonin or a bisphosphonate, dichloromethylene disphosphonate (Cl_2MDP), in rats to successfully block the stimulation of resorption induced by PTH. This resulted in an increase in bone

formation by PTH independently of the need for previous bone resorption. In a more recent study, Hock et al (1992) observed that intermittent PTH administration was significantly more anabolic than continuous PTH. Furthermore, Hock and co workers (1992), using a combination of continuous PTH and Cl_2MDP demonstrated that PTH induced formation independently of its effects on bone resorption.

The combination therapy of nasal calcitonin spray and intermittent PTH injection has been used to increase the trabecular bone mass of osteoporotic patients (Hesch et al 1989). Thus from an anabolic aspect, intermittent treatment with PTH appears to be more beneficial than a continuous infusion.

Osteoblasts, unlike osteoclasts, are capable of directly responding to PTH. This is related to the fact that receptors for PTH have been localized on osteoblasts but not osteoclasts. Isolated osteoclasts do not respond to PTH (McSheehy and Chambers 1986a). It has been postulated that a soluble factor is released from PTH-stimulated osteoblasts which stimulates osteoclast activity. In an attempt to characterise this factor, McSheehy and Chambers (1986b) observed that it was smaller than 1 kD, stable to freeze thawing but inactivated at 65°C after 30 minutes. In contrast, Perry et al (1989), employing similar techniques, isolated a 110 kD protein which was dependent on a divalent cation for retention of its activity. Furthermore, the stimulation of resorbing activity of isolated osteoclasts with this 110 kD factor was not blocked by treatment with indomethacin which therefore indicates that this factor exerts its effects via prostaglandin-independent mechanisms. It must be noted however, that PTH can induce prostaglandin E_2 (PGE_2) in human osteoblasts (MacDonald et al 1984) and enhances IL-1 elevated PGE_2 in rat calvarial osteoblasts (Tatakis et al 1988).

The effects of PTH on osteoblasts at least in the short term are mainly de-differentiating and proliferative changes are variable. These effects may be related

to the initial differentiation status of the osteoblast-like cells used in addition to differences in culture conditions such as cell density, serum concentration and time of exposure. For a summary of effects of PTH on the proliferation of osteoblasts and alkaline phosphatase and collagen type I expression, refer to table 1.1.

Canalis et al (1990) observed that PTH and PTHrP were able to induce the proliferation of cells in fetal rat calvariae. MacDonald et al (1986a) reported that PTH induced the proliferation of human osteoblast-like cells over long-term culture. A proliferative effect on osteoblasts *in vivo* would account for the increase in trabecular bone volume observed in rats following intermittent infusion of PTH.

A characteristic effect of PTH on osteoblast-like cells is the stimulation of the PTH receptor-linked adenylate cyclase and cAMP formation in human (MacDonald et al 1984), murine (Heath et al 1984), rat ROS 17/2.8 (Shigeno et al 1988) and human SaOS-2 (Fukayama and Tashjian 1990) osteoblasts. Many of the effects of PTH are mediated via the stimulation of adenylate cyclase although there are reports of PTH receptors which are coupled to protein kinase C (Hesch et al 1988, Rao and Murray 1989, Mitchell and Goltzman 1990). Recently the cDNA for the PTH-PTHrP receptor has been expressed and cloned in monkey COS-7 cells (Juppner et al 1991). The expressed receptor was capable of binding both PTH and PTHrP with equal affinity and intracellular signaling was linked to the G protein thereby activating adenylate cyclase and cAMP (Juppner et al 1991). The authors reported a significant homology between the PTH-PTHrP and the calcitonin receptor, another G protein-linked receptor. Since both PTH and calcitonin are involved in calcium homeostasis, Juppner and co workers postulated that these novel receptors could represent a new family of G protein-coupled receptors which share functional features that distinguish them from other G protein-linked receptors.

Receptors for PTH have been localised in an embryonic avian bone cell population, the identity of which are likely to be osteoblasts (Pliam et al 1982). Silve et al

(1982) using light microscope autoradiography localised PTH receptors on osteoblasts and progenitor cells but not in osteoclasts or osteocytes in embryonic chick calvariae. Rouleau et al (1988) using a similar isotopic technique *in vivo* detected binding of PTH to a certain population of cells within rat long bones that were distinct from mature osteoblasts. Further studies characterised the target cell for PTH as a slowly dividing cell which may represent an osteoblast precursor although these cells were histologically distinct (Rouleau et al 1990).

1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$)

$1,25(OH)_2D_3$ is the other major calciotropic hormone along with PTH which regulates calcium homeostasis in the intestine, bone and kidney. $1,25(OH)_2D_3$ is considered to represent the hormonally active metabolite of vitamin D_3.

$1,25(OH)_2D_3$, like PTH also stimulates the production of latent collagenase in rat (Shen et al 1988) and mouse (Heath et al 1984) calvarial osteoblast-like cells. Hamilton et al (1984) and Partridge et al (1987) demonstrated that $1,25(OH)_2D_3$ increased plasminogen activator production from UMR106-01 cells.

$1,25(OH)_2D_3$ is also a potent resorbing factor in both fetal rat long bones (Holick and DeLuca 1972, Vargas et al 1990) and neonatal mouse calvariae (Reynolds et al 1973, Linkhart and Keffer 1991) and stimulates the formation of osteoclast-like cells in a variey of long-term marrow culture systems (Ibbotson et al 1984, Roodman et al 1985, MacDonald et al 1987, Takahashi et al 1989, Kurihara et al 1991). Abe et al (1983) observed that $1,25(OH)_2D_3$ was capable of inducing the fusion of mouse alveolar macrophages into multinucleated cells, although these did not resorb bone. Holtrop et al (1981) studied the effects of $1,25(OH)_2D_3$ in thyroparathyroidectomized rats maintained on a vitamin D-deficient diet and demonstrated that independently of PTH, intravenous injection of $1,25(OH)_2D_3$ increased osteoclast number. These osteoclasts had prominent ruffled borders,

clear zones and nuclei. Furthermore, this hormone also increases other osteoclast characteristics such as acid phosphatase and hyaluronate synthesis (Wong 1977). $1,25(OH)_2D_3$ is a potent maturation factor, that is capable of promoting the differentiation of a number of cells along the macrophage lineage. These include human monocytic leukemia U937 cells (Amento et al 1984), HL-60 cells (Bar-Shavit et al 1983, Reichel et al 1987), bone marrow cells (Miyaura et al 1982) and murine myeloid M1 cells (Miyaura et al 1989a). In one of these studies, HL-60 cells differentiated into multinucleated cells which were capable of bone resorption (Bar-Shavit et al 1983). Thus, many lines of evidence suggest that $1,25(OH)_2D_3$ may be involved in osteoclastogenesis by promoting cell differentiation.

$1,25(OH)_2D_3$ also promotes the differentiation (Table 1.2) of the markers of the osteoblast phenotype including upregulation of alkaline phosphatase activity and osteocalcin expression. The effects of $1,25(OH)_2D_3$ on the synthesis of type I collagen are variable and may be species related. Contradictory reports with repect to the effects of $1,25(OH)_2D_3$ on alkaline phosphatase activity may reflect the density and/or maturity status of the osteoblast cell cultures (Majeska and Rodan 1982, Fritsch et al 1985). Furthermore, Owen et al (1991) detected variable effects of $1,25(OH)_2D_3$ on all three parameters in rat osteoblasts. This was dependent on the proliferative and differentiative state of the osteoblasts and also the duration of exposure of the cells to hormone treatment. Osteocalcin, alkaline phosphatase and collagen genes all contain a 5' upstream sequence which contain $1,25(OH)_2D_3$ responsive elements and AP-1 binding sites (Owen et al 1991). Owen et al (1991) observed that during the proliferative phase of osteoblasts, a fos-jun nuclear complex bound a region in the 5' sequence which inhibited the expression of osteocalcin. $1,25(OH)_2D_3$ exposure during cell differentiation may cause the disassociation of this complex thereby initiating transcription of the osteocalcin gene. $1,25(OH)_2D_3$ promotes the production of various other matrix components

from osteoblasts including osteonectin (Thiebaud et al 1989) and osteopontin (Jin et al 1989, Owen et al 1991). Merry et al (1992a) demonstrated that 1,25(OH)$_2$D$_3$ induced transforming growth factor-β (TGFβ) mRNA expression in human osteoblast-like cells. This work was in accordance with a report by Finkelman et al (1991) who observed that rats maintained on a vitamin D-deficient diet lacked TGFβ expression in the matrix. Since osteoblasts are known to release this growth factor, it was proposed that 1,25(OH)$_2$D$_3$ was involved in the promotion of maturation of the osteoblast and elaboration of bone matrix proteins (Finkelman et al 1991). 1,25(OH)$_2$D$_3$ exerts anti-proliferative effects on human osteoblasts (Beresford et al 1986, Evans et al 1990a), rat and mouse calvarial osteoblasts (Chen et al 1983) and ROS 17/2.8 (Majeska and Rodan 1982, Yoneda et al 1984). Skjodt et al (1985) observed a biphasic effect of 1,25(OH)$_2$D$_3$ on human osteoblasts according to the dose used, with high concentrations inhibiting proliferation and low concentrations inducing a small though consistent stimulatory effect.

Receptors for 1,25(OH)$_2$D$_3$ have not been demonstrated in osteoclasts. This may account for the lack of observed effects of 1,25(OH)$_2$D$_3$ on isolated osteoclasts (Chambers and Dunn 1982, McSheehy and Chambers 1987). However the 1,25(OH)$_2$D$_3$ receptor has been demonstrated in various osteoblast lines including MC3T3-E1 (Kurihara et al 1986), UMR series (Partridge et al 1980), mouse calvaria (Chen et al 1979), primary rat (Chen et al 1986), rat calvaria (Kream et al 1977), chick calvaria (Kream et al 1977) and ROS 17/2 (Petkovich et al 1984). Merke et al (1986) demonstrated that the ligand-receptor complex bound to DNA in the nuclear fraction of whole bone preparations, a characteristic of steroid receptors. Receptor levels are regulated by a number of agents including 1,25(OH)$_2$D$_3$ and PTH in UMR 106 cells (Pols et al 1988a, Pols et al 1988b) and by 1,25(OH)$_2$D$_3$ and retinoic acid in MG-63 cells (Mahonen et al 1989).

Vargas et al (1990) characterised the effect of the rat 1,25(OH)$_2$D$_3$-binding protein

in serum which was found to block the action of the hormone as visualised by a decrease in $1,25(OH)_2D_3$-stimulated resorption in fetal rat long bones.

Calcitonin

This small peptide is produced mainly by the C cells in the thyroid.

The characteristic effect of this peptide on bone is the inhibition of basal and stimulated resorption by osteoclasts (Wong et al 1977, Gowen et al 1985, Stern et al 1985, Bertolini et al 1986, deVernejoul et al 1988, Abe et al 1988, Nishihara et al 1989, Fujii et al 1990, Raisz et al 1990, Linkhart et al 1991). Furthermore, calcitonin inhibits the formation of multinucleated cells in long-term marrow culture systems (Ibbotson et al 1984, Takahashi et al 1989, Kurihara et al 1991).

Recently, the cDNA for the calcitonin receptor has been expressed and cloned in monkey COS cells (Lin et al 1991). The authors observed that similar to the PTH-PTHrP receptor, calcitonin receptors were linked to a G protein that subsequently lead to increases of intracellular cAMP upon ligand binding. Lin et al (1991) reported that the calcitonin receptor was a unique structure and only exhibited a degree of amino acid sequence similarity with the PTH-PTHrP receptor. Therefore the calcitonin and PTH-PTHrP receptors appear to represent a new family of G protein-coupled receptors.

Receptors for calcitonin have been observed on osteoclasts (Goldring et al 1988, Shinar et al 1990, Kurihara et al 1991). Chambers et al (1985) was able to demonstrate an inhibition of pit formation by isolated osteoclasts when calcitonin was present and deVernejoul et al (1988) demonstrated a similar inhibitory effect as assessed by ^{45}Ca release from devitalised bone. It appears that the major effect of calcitonin on osteoclasts is the rapid inhibition of osteoclast motility and retraction of pseudopods (Chambers and Dunn 1982, Dempster et al 1987). Dempster et al (1987) observed this effect using synthetic chicken calcitonin on disaggregated rat

osteoclasts, but was unable to demonstrate the same effect on embryonic chicken osteoclasts. This group postulated that native chicken calcitonin may be required for an effect on the chicken system. Several groups have observed an effect known as the 'escape' phenomenon, where the osteoclasts begin to resorb again after a lag period of inactivity in the presence of calcitonin. Fujii et al (1990) observed this effect after two days treatment of calcitonin on fetal mouse forearm bones. Peterlik et al (1985) observed that interferon-γ also had a calcitonin-like inhibitory effect, but differed from calcitonin in that no escape phenomenon was detected. Clinically this phenomenon has been used in combination with PTH for the effective treatment of osteoporotic patients whereby salmon calcitonin is administered for the functional life span of the osteoclast and PTH is given whilst the osteoblasts are actively forming bone (Hesch et al 1989). In a comparative *in vitro* study, Zaidi et al 1987 demonstrated that salmon calcitonin was more potent than human calcitonin with respect to inhibition of bone resorption.

Calcitonin gene-related peptide (CGRP) is not as potent as calcitonin but is capable of decreasing bone resorption (Zaidi et al 1987) and does not exhibit the escape phenomenon (D' Sousa et al 1986).

Calcitonin has also been reported to exert effects on osteoblast-like cells *in vitro*. Klein et al (1991) demonstrated the presence of calcitonin receptors on the osteoblast UMR 106-06 cell line and detected a slight decrease in collagen synthesis and cell proliferation upon addition of calcitonin whereas Farley et al (1991c), using human osteosarcoma SaOS-2 cells observed an increase in cell proliferation and alkaline phosphatase activity. CGRP also exerts effects on osteoblast-like cell lines, increasing intracellular cAMP content (Burns et al 1991). Calcitonin therefore also appears to modulate osteoblast activity although further work is required to gain a clearer understanding.

Glucocorticoids

Glucocorticoids potently inhibit bone resorption (Raisz et al 1972, Mundy and Martin 1982) and decrease osteoclast-like cell formation in long-term marrow culture (Suda et al 1983). In addition, glucocorticoids decrease the transcription of other agents involved in bone resorption such as IL-1 (Lee et al 1988).

The major action of glucocorticoids however is their potent inhibition of bone formation. Glucocorticoids act directly on osteoblast-like cells and these cells have been demonstrated to possess the glucocorticoid receptor (Chen et al 1977). Canalis (1983a) demonstrated that glucocorticoids decrease the transcription of procollagen type I transcription in rat calvarial cells in accordance with a study by Gallagher et al (1983) who also demonstrated that osteocalcin transcription was inhibited by glucocorticoid treatment in human trabecular bone cells. Since Dietrich et al (1979) reported that short-term glucocorticoid treatment caused small increases in collagen, it is possible that these steroids have dual effects on sub-populations of cells at different differentiation stages. A major effect of glucocorticoids is the inhibition of preosteoblast replication which is observed *in vivo* and in most tissue culture systems (Chen et al 1977, Dietrich et al 1979). The overall effect of glucocorticoid treatment therefore results in a decrease in available mature osteoblasts and a subsequent decrease in bone formation.

Prostaglandins (PG)

In a comparative study, PGE_2 was shown to be most potent prostanoid in stimulating bone resorption although PGF, PGA and PGB were all active but with a reduced potency (Dietrich et al 1975). Other groups have also demonstrated a resorptive effect of PG in neonatal mouse calvariae (Katz et al 1983, Peterlik et al

1985) and fetal rat bones (Klein and Raisz 1970, Rodan et al 1981). PGs are thought to mediate the resorption of some agents including epidermal growth factor (EGF), FGF (Tashjian and Levine 1978) and possibly IL-1 (Akatsu et al 1991) although with respect to the latter, PG-independent effects have also been reported (Gowen and Mundy 1986). In long-term bone marrow cultures, the effect of PGE_2 on osteoclast-like cell formation is variable. Ibbotson et al (1984) and Akatsu et al (1991) both observed a stimulatory effect, whereas Roodman et al (1985) observed no effect. Collins and Chambers (1991) reported that whilst PGE_2 alone induced osteoclast-like cell formation in long-term bone marrow culture, it had an inhibitory effect on $1,25(OH)_2D_3$-stimulated osteoclast formation. Hiura et al (1991) observed that PGE_2 was partially responsible for the decreased formation of multinucleated cells in spleen cells of 5-fluorouracil treated mice.

The effect of PGs on isolated osteoclasts was to inhibit resorption (Chambers and Dunn 1982, Chambers et al 1985, deVernejoul et al 1988). deVernejoul et al (1988) observed calcitonin-like effects with PGE_2 in that osteoclast motility was decreased. It is possible that accessory cells are required in the vicinity of the osteoclast in order for PGs to stimulate osteoclastic bone resorption.

Stimulation of bone resorption induced by PGs can also be demonstrated *in vivo*. Tashjian et al (1972) transplanted mice with a PGE_2-producing tumour and observed an increase in plasma calcium levels not observed in control mice or in mice transplanted with non-PGE_2 producing tumours. In addition the cyclo-oxygenase inhibitor, indomethacin, blocked the resorptive effect induced by the tumour.

Norrdin and Shih (1988) found that in dogs with rib fracture, the long-term administration of PGE_2 increased bone formation. Whether this is a direct effect of PGE_2 is not clear since PGE_2 is able to induce the production of growth factors such as IGF-I from osteoblasts that are associated with stimulating bone formation

(McCarthy et al 1991).

PGs themselves are produced by osteoblasts stimulated by a variety of agents such as IL-1, tumour necrosis factor (TNF), thrombin, haptoglobin (Rodan et al 1981, Nolan et al 1983, Gowen 1988, Evans et al 1990b, Frohlander et al 1991) and by mechanical strain (Murray and Rushton 1990). Thus the effects of cytokines such as IL-1 and TNF to stimulate bone resorption may be mediated via the production of PGE_2 from the osteoblast. PGs are also capable of inducing the production of collagenase (Shen et al 1988, Heath et al 1984) and plasminogen activator (Hamilton et al 1984) release from rodent osteoblasts and so this may represent another mechanism whereby PGs exert pro-resorptive effects.

Other effects of PG on osteoblasts include the induction of alkaline phosphatase in MC3T3-E1 cells (Hakeda et al 1985, Kumegawa et al 1984) and stimulation of collagen type I in rat RCT-1 and RCT-3 lines (Ernst et al 1989), chicken pre-osteoblasts (Nagai 1989) and fetal rat pre-osteoblasts (Chyun and Raisz 1984). Prostanoids are therefore able to promote the maturation of the osteoblast phenotype which may account for some of the long-term effects of PGs observed on bone formation.

PGs also promote the proliferation of pre-osteoblasts (Chyun and Raisz 1984), chicken osteoblasts (Van der plas et al 1985) and RCT-1 and RCT-3 rat osteoblast lines (Ernst et al 1989) via IGF-I production.

Local Factors

Transforming growth factor β (TGFβ)

TGFβ represents a member of a growing family of related peptides including TGFβ1, TGFβ2 and bone morphogenetic proteins as well as a variety of other proteins. For a review see Mundy and Bonewald (1991).

TGFβ has been shown to exert variable effects on bone resorption. It stimulates resorption in neonatal mouse calvariae or mouse long bones possibly via the induction of PG (Tashjian et al 1985, Pfeilschifter et al 1988, Dieudonne et al 1991). Others have been unable to demonstrate a resorptive effect of this growth factor in a variety of systems including fetal rat long bones (Pfeilschifter et al 1988) and isolated avian osteoclasts on sperm whale dentine slices (Oreffo et al 1990). Others have demonstrated an inhibitory effect of TGFβ on bone resorption in fetal mouse long bones where osteoclast fusion and migration into the marrow cavity appeared to be inhibited (Dieudonne et al 1991). Furthermore, Pfeilschifter et al (1988) using fetal rat long bones, demonstrated that TGFβ partially or completely blocked the induction of bone resorption by IL-1 and $1,25(OH)_2D_3$ possibly by diverting precursors along a different lineage. This is supported by evidence from long-term marrow cultures where TGFβ is proposed to divert cells away from the osteoclast precursor pool by increasing the number of granulocyte-macrophage colony forming units (Chenu et al 1988). Shinar and Rodan (1990) observed a biphasic effect of TGFβ which was PG and dose-related in long-term mouse bone marrow culture. A low concentration of TGFβ enhanced $1,25(OH)_2D_3$-dependent stimulation of osteoclast-like cell formation via PG generation, whereas at a higher concentration, TGFβ was reported to be directly inhibitory (Shinar and Rodan 1990).

In vivo, persistent or daily injections of TGFβ stimulate woven bone formation and

increase bone thickness in rats and mice and as there were also increased osteoclast numbers, this would suggest that TGFβ may be a coupling factor (Noda and Rodan 1986, Mackie and Trechsel 1990). Furthermore, after 19 days the woven bone was partially replaced by lamellar bone in the mouse (Mackie and Trechsel 1990). The potential involvement of TGFβ in stimulating bone formation was further supported using vitamin D-deficient rats (Finkelman et al 1991). These rats had a reduced skeletal content of TGFβ which resulted in reduced osteoinduction as a consequence of the less mature cells being unable to elaborate bone matrix (Finkelman et al 1991). TGFβ is produced by osteoblasts and chondrocytes in a latent form (Gehron-Robey et al 1987, Oreffo et al 1989, Gelb et al 1990, Pfeilschifter et al 1990a, Pfeilschifter et al 1990b) and some of this can be deposited into the bone matrix. Oreffo et al (1989) postulated that the latent form of TGFβ in the matrix could be activated by osteoclasts during the process of bone resorption. The acidic environment is favourable for the dissociation of latent TGFβ from a binding protein of about 300 kD (Pfeilschifter et al 1990a). Once the TGFβ is activated it may then serve to inhibit further osteoclastic resorption and to promote the formation of osteoblasts and so may function to couple these two phases. This is supported by evidence from the work of Pfeilschifter et al (1990b) who observed that activated TGFβ was transiently chemotactic for osteoblasts.

The proliferative effects of TGFβ on osteoblasts are often biphasic with the concentration of growth factor being critical (Centrella et al 1987, O' Keefe et al 1988). This is also seen for collagen type I synthesis (Centrella et al 1987). Variable effects on alkaline phosphatase are also observed and may be related to cell proliferation (Centrella et al 1987). Centrella and co workers observed that doses of TGFβ that inhibited alkaline phosphatase expression, maximally stimulated proliferation in fetal rat osteoblasts. Conversely, an increase in TGFβ concentration inhibited proliferation and returned alkaline phosphatase to original levels. Noda

and Rodan (1986) using murine non transformed MC3T3-E1 cells reported that alkaline phosphatase levels, which peak when cells are confluent, were inhibited by TGFβ. Overall et al (1989) demonstrated TGFβ-induced release of plasminogen activator inhibitor (PAI) from fibroblasts which would ultimately reduce collagenase activity.

Growth factors

Many growth factors, including transforming growth factor α (TGFα), epidermal growth factor (EGF), platelet derived growth factor (PDGF), insulin-like growth factors (IGFs) and acidic or basic fibroblast growth factors (a or bFGFs) have been demonstrated to have effects on bone. For a review on these refer to Canalis et al (1989), Schmidt and Ernst (1991), and Rodan and Rodan (1991). Growth factors are generally mitogenic although they also may affect the differentiated function of cells. IGFs have been isolated from bone cultures and are produced by osteoblasts. They increase DNA synthesis (Canalis and Lian 1988) and production of collagen type I independently (Canalis and Lian 1988, Pfeilschifter et al 1990c) and also promote the production of alkaline phosphatase. Canalis and Lian (1988) observed that IGF-I and $1,25(OH)_2D_3$ synergistically increased osteocalcin expression in fetal rat calvarial cells. Ernst et al (1989) observed that IGF-I mediated the proliferative effects of oestrogen on RCT-1 and RCT-3 osteoblast like cells. IGFs are therefore capable of inducing cell proliferation and differentiation of cells of the osteoblastic lineage.

TGFα shares extensive amino acid homology with EGF and competes for the same receptor. TGFα, like EGF generally decreases bone formation and stimulates resorption via increased prostaglandin production (Tashjian and Levine 1978, Stern et al 1985, Takahashi et al 1986). Both factors are capable of synergising with IL-1 to further increase bone resorption (Lorenzo et al 1988, Hurley et al 1989).

Furthermore, Takahashi et al (1986) observed that TGFα and EGF both stimulated osteoclast-like cell formation in long-term bone marrow cultures by stimulating the proliferation of precursors. In contrast, FGF, IGF and PDGF in the same experiments, had no effect (Takahashi et al 1986). Collagenolytic enzyme production is also stimulated by EGF in rodent osteoblasts (Hamilton et al 1984, Shen et al 1988). EGF and TGFα both stimulate DNA synthesis in osteoblasts (Ng et al 1983, Hurley et al 1989) and reduce the synthesis of collagen (Ibbotson et al 1986, Hurley et al 1989) and alkaline phosphatase (Ibbotson et al 1986).

FGFs exert mitogenic effects on bone cells (Canalis et al 1988, Canalis and Lian 1988, Globus et al 1989) and both aFGF and bFGF are synthesised *in vitro* by fetal bovine bone cells which have characteristics of the osteoblast phenotype such as osteocalcin expression (Globus et al 1989). FGFs are present in the matrix and it is thought that FGF, released from the inactive complex of FGF bound to heparan sulphate containing proteoglycans, is able to promote the cell replication of osteoblasts (Globus et al 1989). Inhibition of the mature osteoblast phenotype, including down regulation of alkaline phosphatase and collagen synthesis (Canalis et al 1988) and a decrease in PTH-stimulated adenylate cyclase accompanies the stimulation of osteoblast proliferation by FGF. FGF stimulates bone resorption apparently by a similar mechanism to EGF (Tashjian and Levine 1978).

PDGF is also present in the matrix and increases bone DNA synthesis in fetal rat calvariae (Canalis 1987b). PDGF stimulates PG synthesis which leads to an increase in bone resorption in a manner similar to EGF, however this factor is mitogenic for mesenchymal cells and may therefore attract cells of the osteoblast lineage to the vicinity of the resorption lacunae thereby promoting the coupling effect.

Colony stimulating factors (CSFs)

The CSFs represent a group of peptides which are involved in various stages of hematopoiesis and include granulocyte-macrophage-CSF (GM-CSF), granulocyte-CSF (G-CSF), macrophage-CSF-(M-CSF) and IL-3, also known as multi-CSF. For a review refer to Horowitz and Jilka (1991).

The most characterised function of IL-3 is to induce the proliferation and differentiation of early pluripotent stem cells however, IL-3 also stimulates the production of osteoclasts from purified murine stem cells in the presence of stripped metatarsal bones (Scheven et al 1986).

In long-term marrow culture systems, the effect of GM-CSF on formation of osteoclast-like cells has been variable, and may be dependent upon the maturity of the culture system used. MacDonald et al (1986b) reported that GM-CSF stimulated osteoclast-like cell production by baboon long-term marrow culture, whereas Lorenzo et al (1987) and Shinar et al (1990), using long-term mouse marrow culture failed to observe any effects of GM-CSF.

GM-CSF is produced by osteoblasts stimulated with lipopolysaccharide (LPS) or PTH (Horowitz et al 1989, Weir et al 1989) and has been reported to increase the proliferation of osteoblasts (Dedhar et al 1988, Evans et al 1989a).

M-CSF stimulates the proliferation and differentiation of more mature hematopoietic cells, monocytes and macrophages. There are also conflicting reports as to whether M-CSF induces osteoclast formation in long-term bone marrow cultures. *In vivo* however, in osteopetrotic mice, there is extensive evidence to show the importance of M-CSF in osteoclast biology since the deficiency of osteoclasts in this condition can be rectified following administration of M-CSF (Felix et al 1990). This would suggest that at least in the mouse, M-CSF is involved in osteoclastogenesis. In addition, MacDonald et al (1986b) observed that M-CSF increased the formation of osteoclast-like cells in long-term baboon marrow culture.

Osteoblasts when stimulated with bacterial endotoxin, IL-1, PTH, and PTHrP produce M-CSF (Sato et al 1986, Elford et al 1987, Felix et al 1989, Horowitz et al 1990) and therefore these cells may influence osteoclast maturation and activity.

G-CSF induces the proliferation of granulocytic precursors. Its effects tend to divert the multipotential precursors away from the osteoclast lineage and towards granulocytes. The precursor for granulocytes does not give rise to osteoclasts (Hattersley et al 1991).

Under certain conditions, some osteoblast lines have been shown to produce G-CSF. Primary cultures of rat osteoblasts can produce G-CSF in the absence of IL-1 (Horowitz, unpublished observations). The majority of work however, shows that production of G-CSF is not a common feature of osteoblasts.

Interleukin 1 (IL-1)

Two separate gene products of IL-1 exist these being IL-1α and IL-1β. These represent two related proteins with a similar broad spectrum of activities which are reviewed by Dinarello (1988, 1989).

The effects of IL-1 as a potent resorbing agent have long been recognised. Initial studies using conditioned media from stimulated lymphocytes (Horton et al 1972) and mononuclear cells (Mundy et al 1977) demonstrated that factors produced from the cells could stimulate bone resorption in fetal rat long bones. The first indication that the potent soluble resorbing factor was due to IL-1 activity was demonstrated by Gowen et al (1983) and Dewhirst et al (1985). The factor was identified by these groups to be approximately 17 kD and its NH_2-terminal sequence identical to IL-1β. Subsequent to these studies, several groups have demonstrated the stimulation of bone resorption by IL-1 in neonatal mouse calvariae (Gowen and Mundy 1986) and fetal rat calvariae (Dewhirst et al 1990). Nishihara et al (1989) observed that the membrane bound form of IL-1 was also capable of inducing bone

resorption. Further studies *in vivo* also demonstrated similar effects (Konig et al 1988, Sabatini et al 1988, Sato et al 1989).

Although IL-1 clearly stimulates bone resorption, there appears to be conflicting evidence as to whether IL-1 exerts its resorptive effects via the generation of PGs. Some groups observed that IL-1 induced bone resorption via a PGE_2-independent mechanism (Gowen and Mundy 1986, Dewhirst et al 1987, Amano et al 1988) whereas others demonstrated prostaglandin-dependent effects (Lorenzo et al 1988). Many groups have observed that IL-1-stimulated bone resorption requires partial dependence of PGs (Dewhirst et al 1987, Stashenko et al 1987a, Nishihara et al 1989). A possible reason for the discrepancy may lie in the fact that different bone resorption systems were utilized. Unlike calvariae, long bones contain chondrocytes which are capable of releasing PGs (Stashenko et al 1987a, Dewhirst et al 1987). This is unlikely to be the full explanation however, as IL-1-stimulates osteoblasts from several species to produce prostaglandins. These include human (Gowen 1988, Evans et al 1989b, Evans et al 1990b) rat calvarial (Tatakis et al 1988) and MC3T3-E1 murine osteoblasts (Ikeda et al 1988). It is likely that prostaglandin-dependent and -independent effects are observed during the resorption of bone stimulated by agents such as IL-1 and TNF, and may be due to the concentrations of resorption-stimulating factors present. The fact that IL-1 synergises with other agents including transforming growth factor α (TGFα) (Lorenzo et al 1988, Hurley et al 1989), PTH (Dewhirst et al 1987), epidermal growth factor (EGF) (Lorenzo et al 1988), PTHrP (Sato et al 1989), tumour necrosis factor α and β (TNF) (Stashenko et al 1987a), interleukin 6 (IL-6) (Garrett et al 1990) and stimulators of cAMP (Dewhirst et al 1990) to stimulate bone resorption further supports this view, as prostaglandin involvement in the above is variable.

One of the possible ways by which IL-1 can stimulate bone resorption is via an

increase in the formation and number of osteoclasts. This is evident in long-term bone marrow cultures that can be stimulated to form multinucleated cells with osteoclast-like characteristics (Takahashi et al 1989). Pfeilshifter et al (1989) demonstrated that the IL-1-induced formation of these cells in long-term human bone marrow culture occurred via a prostaglandin-independent mechanism. Kurihara et al (1991) observed that IL-1β was capable of inducing osteoclast formation from both early and late precursors within the culture. Presumably, IL-1 increased the proliferation of the early precursors and subsequently induced their fusion. Others have reported that IL-1 is capable of maintaining the number of osteoclasts which would normally decline in neonatal mouse calvariae (Garrett and Mundy 1989). They postulated that IL-1 acted on the later stages of osteoclast formation since inhibitors of DNA synthesis did not alter the IL-1 effects.

IL-1, by reducing proteoglycan content, exerts other degradative effects on bone. The reduction of proteoglycans by IL-1 may be achieved by decreased proteoglycan synthesis or increased breakdown and was observed in murine patella chondrocytes (Van Den Berg et al 1988), human articular cartilage (Nietfeld et al 1990) and bovine nasal cartilage explants (Smith et al 1991). In addition, an intra articular injection of IL-1 into rabbit knee joints decreased proteoglycan content *in vivo* (Henderson et al 1991). Gowen et al (1984) and Rifas et al (1989) observed that IL-1 increased the secretion of collagenase and proteoglycanase in human articular chondrocytes but was unable to demonstrate this in human osteoblasts. Preliminary evidence by Bord et al (1991) may indicate that this is not the case as they demonstrated collagenase by immunofluorescence in human osteoblasts. Osteoblasts from other species such as rat have been shown to produce the latent form of collagenase upon stimulation with IL-1 (Shen et al 1988). Evans et al (1989b, 1990b) demonstrated that human osteoblasts can be induced by IL-1 to produce plasminogen activator which, via an enzyme cascade, is capable of

cleaving latent collagenase to the active form. By the secretion of these enzymes, it is possible to envisage how the stimulation of osteoblasts by IL-1 can degrade the upper collagenous layer thereby exposing the underlying calcified matrix to the osteoclasts. However, it must be stressed that osteoblasts can also produce a number of inhibitors such as plasminogen activator inhibitor (PAI), tissue inhibitor of metalloproteinases (TIMP) and collagenase inhibitor which are all capable of inactivating these matrix degradative enzymes and so highlights the complexity of the potential mechanisms.

Other reports of the effects of IL-1 on osteoblasts demonstrate that this cytokine opposes the basal or $1,25(OH)_2D_3$-stimulated differentiation of osteoblasts by reducing collagen type I production, alkaline phosphatase expression and osteocalcin synthesis (table 1.3). However, the length of incubation period (Canalis 1986) and presence of serum (Hanazawa et al 1986) are influential in these actions.

Another influence of IL-1 is the induction of cell proliferation of osteoblasts. This has been observed in fetal rat calvarial (Canalis 1986, Smith et al 1987), human (Gowen et al 1985, Gowen 1988, Evans et al 1989b, Evans et al 1990b) and MC3T3-E1 (Ikeda et al 1988) osteoblasts. These anti-differentiation, pro-proliferating effects may therefore serve to increase the immature osteoblast precursor pool in preparation for the formation phase and has lead to the proposition that IL-1 may exist as a coupling factor in the remodelling process. An *in vivo* study by Boyce et al (1989) supports this hypothesis as subcutaneous injection of IL-1 over mouse calvariae resulted in the increased number of osteoclasts and resorption pits in the short term. However long-term effects include periosteal cell proliferation, with the resorbed bone being subsequently replaced by new woven bone.

Some evidence would suggest that the effects of IL-1 on osteoclasts are not direct

(Thomson et al 1986), rather, the presence of IL-1-stimulated osteoblasts are required. Thomson et al (1986) postulated that osteoblasts stimulate osteoclasts to resorb bone via the release of a soluble factor induced by IL-1 from the osteoblasts. This provides evidence that IL-1 actions on osteoclasts are not direct. IL-1 is capable of inducing the release of many cytokines and growth factors from osteoblasts (Gowen et al 1990, Merry et al 1990, Littlewood et al 1991b, Linkhart et al 1991b) and other cells present within the bone microenvironment. These factors may in turn exert actions on the bone cells and thereby create a potential complex network of synergistic and antagonistic interactions. Furthermore, many of these factors are able in turn to induce the production of IL-1 from cells within the bone microenvironment (Merry et al 1990, Kurihara et al 1990).

Receptors for IL-1 have been demonstrated on a number of different cell types including osteoblasts (Bird and Saklatvala 1986, Shen et al 1990), T-lymphocytes (O'Neill et al 1990), fibroblasts (O'Neill et al 1990, Dower et al 1986) and B-cell lines (Bensimon et al 1989). Dower et al (1986) observed that the receptor for IL-1 on murine and human cells were identical and that both α and β forms of IL-1 bind to the same receptor. The majority of evidence indicates the presence of two receptors; a high affinity 55 kD and a low affinity 80 kD receptor (reviewed by Dinarello et al 1989).

Antagonists for the action of IL-1 function by competing for the 80 kD receptor (Hannum et al 1990, Carter et al 1990, Seckinger et al 1987a, 1987b, Mazzai et al 1990) rather than by binding IL-1 to inactivate it. Natural inhibitors for IL-1 were first isolated from the urine of monocytic leukemia patients (Seckinger et al 1987a) and from human monocytes stimulated with IgG (Hannum et al 1990). The cDNA was subsequently obtained from U937 cells (Carter et al 1990) and monocytes (Eisenberg et al 1990) and the recombinant protein characterised and called interleukin-1 receptor antagonist protein (IRAP). Seckinger et al (1990) observed

that IRAP was capable of blocking IL-1-stimulated bone resorption in both fetal rat long bones and in mouse calvariae. However, higher concentrations of IRAP were required to block the induction of bone resorption in mouse calvariae compared to the fetal rat long bones indicating a further difference between these two bone resorption systems. IRAP also antagonised IL-1-stimulated PGE_2 production in synovial cells (Arend et al 1990) and collagenase production in chondrocytes induced with IL-1 (Arend et al 1990, Smith et al 1991). IRAP is also an effective IL-1 inhibitor *in vivo*, blocking the decreases in proteoglycan content induced by IL-1 in rabbit knee joints (Henderson et al 1991).

The overall effect of IL-1 on bone *in vivo* is likely to be dependent upon the relative concentrations that exist between IL-1 and the IL-1 inhibitors.

Tumour necrosis factor (TNF)

TNF shares many similar activities with IL-1 although it is substantially less potent in these respects. It also exists as two separate gene products, those being TNFα and TNFβ and the proteins only share a 30% amino acid homology (reviewed by Beutler and Cerami 1987).

Both TNFα and TNFβ have been demonstrated to induce bone resorption in fetal rat long bones (Bertolini et al 1986, Stashenko et al 1987a) and mouse calvariae (Gowen and Mundy 1986). Both TNFα and β were less potent than IL-1 in inducing bone resorption in a comparative study (Stashenko et al 1987a). This is also reflected in the ability to stimulate prostaglandin production by osteoblasts (Gowen 1988). TNF exhibits a synergism with IL-1 in inducing bone resorption (Stashenko et al 1987a) which may be an important influence in bone metabolism since they are both capable of inducing each others synthesis in a range of cell types. TNF also induces bone resorption *in vivo* (Garrett et al 1987, Konig et al 1988).

The mechanism of bone resorption induced by TNF appears to be similar to IL-1. Bertolini et al (1986) observed that TNF increased the number of osteoclasts and decreased the production of mineralized bone matrix. Both forms of TNF were active in inducing multinucleated osteoclast-like cell formation in the long-term human marrow culture (Pfeilschifter et al 1989). Preliminary *in situ* hybridisation studies indicate the presence of TNFα and TNFβ in a population of osteoclast precursors (Qi et al 1991) although further studies are necessary to substantiate this evidence. Thomson et al (1987) observed that the effects of TNF on isolated osteoclasts were not direct and were probably mediated by the release of a soluble factor from osteoblasts. TNF is known to be capable of inducing the production of a range of growth factors from many different cell types, however, the identity of the soluble factor described by Thomson et al (1987) is still unresolved.

Another possible degradative effect of TNF on bone matrix may be via the decrease in proteoglycan content observed in porcine articular cartilage and bovine nasal septum (Saklatvala 1986). Furthermore, TNF decreases the synthesis of GAGs in rabbit chondrocytes (Enomoto et al 1990) and increases collagenase production by rat calvarial osteoblasts (Shen et al 1988).

The effects of TNF on osteoblasts are comparable to IL-1, although TNF is less potent in these respects (Gowen 1988). TNF appears to decrease the basal or $1,25(OH)_2D_3$-induced expression of mature phenotypic markers of the osteoblast (table 1.4) whilst promoting the proliferation of human (Gowen et al 1988, Gowen 1988) and rat (Centrella et al 1988) osteoblasts although contrary results are reported (Nanes et al 1989).

Unlike IL-1, TNFα decreases the PTH-induced generation of cAMP in the human osteosarcoma cell line, SaOS-2, and in primary rat osteoblasts. This appeared to be a specific effect since it did not modify PGE_2-induced cAMP generation. Therefore, TNF may alter the effects of PTH on osteoblasts (Shapiro et al 1990).

Receptors for TNF have recently been cloned (Smith et al 1990). Both TNFα and TNFβ bind to these receptors of which two (55 kD and 75 kD) have been isolated. The purified 55 kD receptor was expressed as the recombinant protein and was demonstrated to bind TNF (Loetscher et al 1990). Thoma et al (1990) observed that for some TNF-induced activities, both receptors were necessary to elicit certain TNF responses such as TNF-induced cytotoxicity and enhancement of IL-2 receptor expression in T cell lines. TNF binding proteins have been isolated from the serum of cancer patients (Schall et al 1990). Loetscher et al (1990) demonstrated that the TNF binding protein did not arise from a second gene that may encode a soluble inhibitor.

The TNF binding protein was partially sequenced and shown to be a soluble form of the 55 kD receptor (Schall et al 1990, Smith et al 1990). By binding to TNF it was able to inactivate the cytokine. Engelman et al (1990) isolated two proteins that specifically bound TNF in human urine. Both decreased the cytotoxic effect of TNF *in vitro* and bound TNFα more efficiently than TNFβ. This group also demonstrated that these immunologically distinct proteins were structurally related to the TNF receptor. They may possibly represent soluble forms of the two receptors although amino acid analysis is required to unequivocally clarify this (Engelman et al 1990). Nophar et al (1990) also demonstrated the presence of a binding protein in urine which exhibited significant homology with the 80 kD receptor but was in fact a soluble form of the 55 kD receptor. *In vivo* studies demonstrated that TNF binding proteins are transiently increased following intravenous infusions of TNF and IFNγ of cancer patients. This may represent a mechanism for reducing active TNF levels in disease.

Leukemia inhibitory factor (LIF)

LIF was initially isolated and characterised by its ability to suppress the proliferation of myeloid leukemia cells M1 (Hilton et al 1988). With the recent biochemical and molecular characterisation of LIF, it became apparent that HILDA (human interleukin for DA cells) (Moreau et al 1988), DIA (differentiation inhibitory activity) (Smith et al 1988) and DIF (differentiation inducing factor) (Abe et al 1989) were all the same peptide. It is evident that the effects of LIF on many different cell types are pleiotropic, LIF can exert differentiation inhibitory properties, such as maintaining pluripotent embryonic stem cells in an immature, proliferative state (Williams et al 1988, Smith et al 1988). Conversely this factor can also stimulate the differentiation of other cells for example M1 cells towards the macrophage lineage (Hilton et al 1988, Metcalf 1989, Miyaura et al 1989a). Williams et al (1988) proposed that distinct intracellular signalling pathways exist in these two cell types resulting in opposing differentiation effects. LIF possibly regulates the proliferation and differentiation of hematopoietic precursors (Le et al 1990). Murray et al (1990), detected mRNA and protein for both LIF and IL-6 in mouse blastocysts and proposed that these factors exert an early hematopoietic role in mouse embryogenesis.

It is possible that LIF could direct pluripotent stem cells towards the osteoclast lineage and may therefore exert a permissive role on bone resorption. Abe et al (1988) observed that purified LIF from differentiated MC3T3-E1 osteoblast-like cells increased resorption and osteoclast number in mouse calvaria, probably via the action of prostaglandins. This study was confirmed by the work of Reid et al (1990), however contradictory reports are also published. In a complex study Van Beek et al (1991) studied the effect of LIF on resorption in mouse metacarpals and radii of different ages. LIF decreased bone resorption both in metacarpals, where osteoclasts precursors are present, and in older metacarpals and radii containing

mature osteoclasts. LIF appeared to be exerting its effects by blocking osteoclast formation as TRAP positive cells in the matrix were absent. LIF may well have been directing hematopoiesis along a different, but related lineage such as the macrophage lineage. Variable results have also been obtained in long-term mouse bone marrow cultures, with LIF either exerting no effect (Shinar et al 1990) or inducing osteoclast formation (Abe et al 1988). Differences may exist in the maturity of the marrow cells within the culture.

Osteoblasts, but not osteoclasts, possess receptors for LIF (Allan et al 1990, Rodan et al 1990) and also respond to LIF in a number of ways. Two groups independently observed that LIF increased the retinoic acid-induced alkaline phosphatase in osteoblast-like cells, UMR 201 (Allan et al 1990) and RCT-1 (Rodan et al 1990). Evans et al (1992) observed that LIF exerted no effect on basal or $1,25(OH)_2D_3$-stimulated alkaline phosphatase levels. However, when $1,25(OH)_2D_3$ induction of alkaline phosphatase activity was low, LIF was able to potentiate the effects of $1,25(OH)_2D_3$ (Evans et al 1992). In contrasting studies, LIF was reported to reduce alkaline phosphatase activity in MC3T3-E1 cells (Hakeda et al 1991, Noda et al 1990). The differentiation state of the various osteoblast lines may have some influence on these results. LIF has also been reported to inhibit collagen synthesis (Rodan et al 1990, Noda et al 1990), proliferation (Noda et al 1990), and increase osteopontin mRNA (Noda et al 1990). LIF is produced by a number of osteoblast lines including MC3T3-E1 cells (Abe et al 1988), UMR series (Allan et al 1990) and human osteoblasts (Evans et al 1990c). Production of LIF by osteoblast-like cells may indicate a potential paracrine/autocrine function of the cytokine on bone cell metabolism.

Interleukin 6 (IL-6)

IL-6 has been cloned from a human T cell line (Hirano et al 1986), human monocytes (Brakenhoff et al 1987), murine T cells and murine bone marrow (Chiu et al 1988, Van Snick et al 1988). The recombinant IL-6 protein from human and murine cells was expressed and found to possess a degree of homology; 65% at the nucleotide level and 41% at the amino acid level. Recombinant murine IL-6 was demonstrated to contain 211 amino acids (Chiu et al 1988, Van Snick et al 1988) whereas the recombinant human IL-6 consisted of 212 amino acids (Hirano et al 1986). IL-6 structure is unrelated to most other cytokines, however, Simpson et al (1988) observed that the disulphide pattern was identical to granulocyte colony stimulating factor (G-CSF) and therefore IL-6 may have evolved from a common gene.

IL-6 exerts pleiotropic actions which have been characterised by many different laboratories and therefore this cytokine was originally known by a variety of names according to its function on a particular cell type. IL-6 is a potent plasmacytoma and hybridoma growth factor, therefore several groups called it plasmacytoma growth factor (PCTGF) or hybridoma growth factor (HGF) (Simpson et al 1988, Reis et al 1988, Van Damme et al 1987a, Van Damme et al 1987c). IL-6 was designated B cell stimulatory factor-2 (BCSF-2 or BSF-2) as it exhibited potent effects on B cells (see below). Others observed an antiviral action of IL-6 hence it was also known as IFN-β2 although this activity is controversial and most groups are unable to reproduce these findings.

IL-6 is produced during an inflammatory response and has many well characterised activities during inflammation including the stimulation of acute phase proteins (Andus et al 1987, Baumann et al 1987) and effects on B and T cells (reviews Kishimoto and Hirano 1988, Akira et al 1990a). IL-6 stimulates the final maturation of mitogen-stimulated B cells into immunoglobulin producing cells

(Hirano et al 1986, Emilie et al 1988, Muraguchi et al 1988, Clark and Shu 1990).

IL-6 exerts IL-1-like effects on mitogen stimulated- thymocytes and T lymphocyte subsets by inducing proliferation of these cells (Garman et al 1987, Helle et al 1988a, Le et al 1988, Houssiau et al 1988b, Habetswallner et al 1988, Lotz et al 1988, Hodgkin et al 1988, Ceuppens et al 1988, Tosato and Pike 1988, Okada et al 1988, Takai et al 1988). There is controversy as to whether IL-2 involvement is essential or not; and it is likely that IL-2 dependent and independent pathways exist (Hodgkin et al 1988, Le et al 1988) depending on the different T cell subsets. Furthermore, a synergistic proliferation of T cells with IL-1 and IL-6 is also observed in some cases (Helle et al 1988a, Houssiau et al 1988b). In contrast to the late actions of IL-6 in the B cell response, it appears as though this cytokine acts at the early stages of T cell proliferation (Ceuppens et al 1988, Tosato and Pike 1988) and this may be due to the levels of IL-6 receptor expressed by resting B and T cells (discussed in chapter 6).

IL-6 is involved in acute phase response by regulating the production of acute phase proteins from hepatocytes (Andus et al 1987, Baumann et al 1987). IL-6 has been demonstrated to increase the majority of acute phase proteins such as C reactive protein and haptoglobin whilst decreasing albumin production in human hepatoma cells (Morrone et al 1988). Many groups have studied the effect of IL-6 on acute phase protein release from a variety of hepatocytic lines including primary rat hepatocytes (Andus et al 1988, Gauldie et al 1987), human hepatoma cells (Morrone et al 1988, Ramadori et al 1988, Gauldie et al 1987, May et al 1988a), and adult human hepatocytes (Castell et al 1989). Although IL-1, TNF and LIF are able to induce a subset of acute phase proteins, IL-6 was able to modulate a much larger range, making it the most important mediator of the acute phase response (Castell et al 1989).

The mechanism by which IL-6 regulates acute phase protein synthesis has recently

been elucidated (Poli and Cortese 1989). IL-6 has been shown to induce a nuclear protein, IL-6 DNA binding protein (IL-6DBP) in a human hepatoma cell line that binds to the promoter region of acute phase genes. This region is known as the IL-6 responsive element and binding of the nuclear protein induces transcription of the acute phase protein mRNA. Akira et al (1990b) also purified a nuclear protein, nuclear factor IL-6 (NF-IL6). The C terminal region of NF-IL6, which is essential for DNA binding, is highly homologous with a liver specific transcription factor, C/EBP (Akira et al 1990b). The significance of this has yet to be determined. Akira and co-workers also demonstrated that the expression of NF-IL6 could be induced by LPS, IL-1 and IL-6 and that NF-IL6 bound to gene regulatory regions for various acute phase proteins. Other workers have also established the presence of IL-6 responsive elements in the promoter region of certain acute phase genes (Poli and Cortese 1989, Oliviero and Cortese 1989, Ito et al 1989, Marinkovic and Baumann 1990,.Baumann et al 1990a). Yap et al (1991) observed that IL-1, TNF and IL-6 all interact to enhance or inhibit certain acute phase proteins in human hepatocytes. During an acute phase response, many of these factors will be increased and therefore their combination may be critical for the appearance of certain acute phase proteins.

During certain acute conditions IL-6 levels are elevated in the serum, such as systemic lupus erythematosus (SLE) (Swaak et al 1989, Israeli et al 1991, Stuart et al 1991), alcoholic hepatitis (Sheron et al 1991), renal transplant recipients (Van Oers et al 1988), surgical intervention (Nishimoto et al 1989, DiPadova et al 1991), severe burns (Nijsten et al 1987) and fever (Helle et al 1988b, Lesnikov et al 1991). In some of these studies, IL-6 levels in the serum have been reported to correlate with acute phase proteins, in particular CRP although evidence is highly subjective. Houssiau et al (1988a) demonstrated the presence of raised IL-6 levels in cerebro-spinal fluid of patients with acute infections of the central nervous system such as

meningitis and tuberculosis.

Rheumatoid arthritis is a chronic inflammatory condition characterised by massive cellular infiltration and proliferation of the synovial membrane to form a pannus that grows over and constitutes the erosion of cartilage and bone via release of destructive factors. Several groups have demonstrated raised IL-6 levels in synovial fluid, synovial cells and serum of patients with rheumatoid and other inflammatory arthritides when compared to osteoarthritic patients (Swaak et al 1988, Hirano et al 1988, Houssiau et al 1988c, Nietfeld et al 1990). There are contrasting studies, such as those of Guerne et al (1989) who demonstrated that there was no difference between IL-6 production from rheumatoid and osteoarthritic synoviocytes.

Raised serum levels of IL-6 are observed in the disease psoriasis, and IL-6 promotes the symptoms of this disease characterised by extensive epidermal proliferation and inflammation (Grossman et al 1989, Neuner et al 1991). Muller et al (1991) demonstrated that $1,25(OH)_2D_3$ or its analogue MC903 decreased the production of IL-6 in LPS-stimulated mononuclear cells and blocked IL-6-driven murine thymocyte proliferation. Morimoto et al (1986) has observed that $1,25(OH)_2D_3$ and MC903 are effective in the treatment of psoriasis and so Muller et al (1991) surmised that these agents may be acting by blocking the effects of IL-6. Arthritis is often associated with psoriasis and some groups are now investigating the role of IL-6 in pathogenesis of psoriatic arthropathy.

Many groups have observed increased IL-6 expression in various carcinomas (Hirano et al 1987, Erroi et al 1989, Tabibzadeh et al 1989), sarcoma cells (Miles et al 1990) and leukemic cells (Biondi et al 1989). It is possible that high levels of IL-6 from carcinomas may account for the raised acute phase proteins associated with malignancy, although more evidence is required.

Effects of IL-6 within the bone microenvironment have only recently been studied and as this constitutes a large portion of this thesis; the discussion in this respect is left to later chapters.

For general reviews on IL-6 activity, see Wong and Clark (1988), Hirano et al 1990.

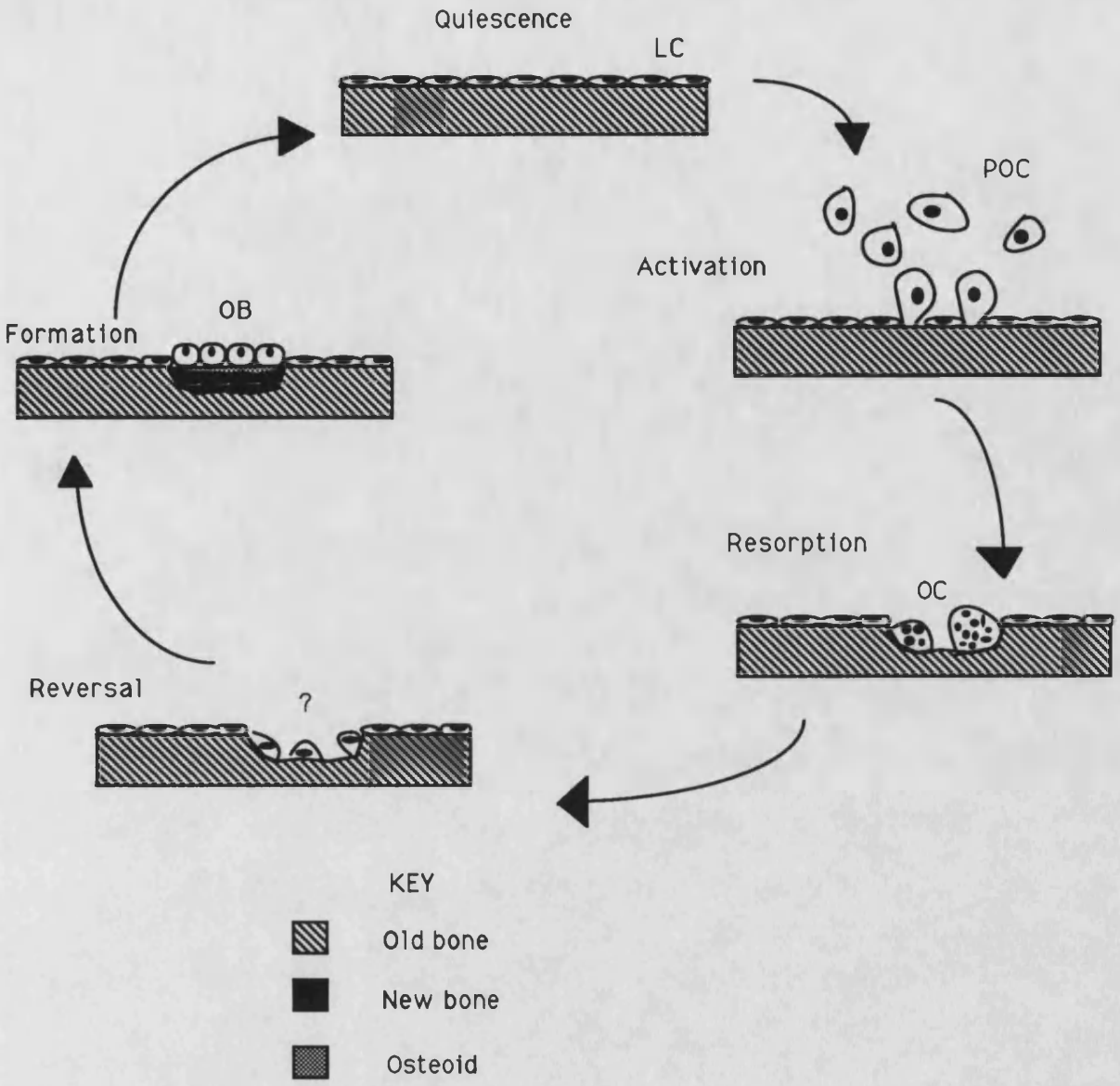


Fig. 1.1 **<u>The remodelling sequence in normal human adult bone.</u>**
LC - lining cell. POC - preosteoclast.
OC - osteoclast. OB - osteoblast.

Table 1.1 The effect of PTH on various osteoblast parameters

		Alkaline phosphatase activity	Collagen Type I production	proliferation
Human osteoblasts	+			MacDonald et al 1986a
	-			
Chicken osteoblasts	+			Van der Plas et al 1985
	-			
Mouse calvarial osteoblasts	+			
	-	Wong et al 1977	Wong et al 1977	
ROS 17/2	+			
	-		Kream et al 1986	
UMR 106	+			
	-			Partridge et al 1985
Fetal rat calvaria	+		Canalis et al 1990	Canalis et al 1990
	-	Canalis 1983b	Kream et al 1980 Canalis et al 1990	

KEY

+ Stimulatory effect
- Inhibitory effect

Table 1.2 The effect of $1,25(OH)_2D_3$ on various osteoblast parameters.

	Alkaline phosphatase activity		Osteocalcin production		Collagen Type I production	
Human osteoblasts	+	Beresford et al 1986 Evans et al 1990a	+	Skjodt et al 1985 Evans et al 1990a	+	Beresford et al 1986
Mouse osteoblasts	-	Wong et al 1977			-	Wong et al 1977
Chick osteoblasts	-	Broess et et 1989	-	Broess et et 1989	-	Broess et et 1989
ROS 17/2	+	Grigoriadis et al 1986 Manolagas et al 1981	+	Lian et al 1988 Price and Baukol 1980 Fraser and Price 1990	-	Kream et al 1986
ROS 17/2.8	+	Catherwood et al 1988 Yoneda et al 1984				
	+/-	Majeska and Rodan 1982				
Rat osteoblasts	+/-	Owen et al 1991	+/-	Owen et al 1991	+/-	Owen et al 1991
MC3T3-E1	+	Kurihara et al 1986 Kurose et al 1990			+	Kurihara et al 1986
Fetal rat calvaria	-	Canalis 1983b			-	Canalis 1983b Rowe and Kream 1982
Chick medullary organ culture					-	Harrison and Clark 1986

KEY

+ stimulated by $1,25(OH)_2D_3$
- inhibited by $1,25(OH)_2D_3$
+/- stimulated and inhibited by $1,25(OH)_2D_3$

Table 1.3

Inhibitory effect of IL-1 on basal and $1,25(OH)_2D_3$-stimulated osteoblast parameters.

Osteoblast model / Decreased parameter	Alkaline phosphatase activity	Osteocalcin production	Collagen type I production
Human osteoblasts	Gowen 1988 Evans et al 1990b	Beresford et al 1984a Gowen 1988 Evans et al 1990b	Beresford et al 1984a
Rat calvarial osteoblasts	Stashenko et al 1987b		Stashenko et al 1987b Smith et al 1987 Canalis 1986
MC3T3-E1 murine osteoblasts	Ikeda et al 1988		Ikeda et al 1988

Table 1.4

Inhibitory effect of TNF on basal and $1,25(OH)_2D_3$-stimulated osteoblast parameters

osteoblast model / decreased parameter	Alkaline phosphatse activity	Osteocalcin	Collagen type I production
Human osteoblasts		Gowen 1988	
ROS 17/2.8 rat osteoblasts	Bertolini et al 1986	Nanes et al 1991	Nanes et al 1989
Fetal rat osteoblasts			Centrella et al 1988
Fetal rat bone/ calvaria	Centrella et al 1988		Smith et al 1987 Bertolini et al 1986

CHAPTER 2

METHODS

Human osteoblast-like cell culture

Human trabecular bone, obtained from femoral heads or above-knee amputations was dissected, in a sterile environment, into pieces of approximately 0.3 cm^3. This was placed in a sterile container and washed extensively with phosphate buffered saline (PBS) to remove non-adherent bone marrow tissue and fatty deposits. Any soft connective tissue was then clearly visible and could be carefully removed after the washing procedure. The PBS was then replaced with Eagles Minimal essential medium (EMEM) supplemented with 10% (v/v) heat inactivated FCS, 100 U/ml penicillin, 100 ug/ml streptomycin and 2 mM L-glutamine (EMEM + 10% FCS). The bone pieces were incubated over night in 5% CO_2 at 37°C in a humidified atmosphere to allow full separation of any remaining fat deposits. After this time, the medium was removed and the bone chips plated out at 6 - 8 explants per 9 cm tissue culture petri dish in 10 ml fresh EMEM + 10% FCS. The medium was changed once a week and cells migrated from the bone explants after two weeks of initiation of the culture. Cells became confluent after four to six weeks and at this stage, could then be passaged for experimental purposes.

Cell Passaging

The human osteoblast-like cells were passaged into 24, 48 or 96 well plates. The explants of bone were removed and could be added to fresh 9 cm tissue culture petri dishes for further culture. The medium was removed from the petri dish and the cell layer washed once with PBS. Trypsin-EDTA (2.5 ml) was added to the cell layer and the plate returned to the incubator at 37°C for 5 - 15 minutes. The cell layer then became detached and EMEM + 10% FCS was added to neutralise the trypsin from proteolytically damaging the cells. The cells were centrifuged at 1000 rpm for

7 minutes and the pellet was resuspended gently in 2 ml of EMEM + 10% FCS with a syringe and needle. The suspension was adjusted to 10 ml with EMEM + 10% FCS and cells counted on a haemocytometer. The cells were then plated out into the multi well plates at the appropriate cell density for the particular experiment.

ROS 17/2.8 culture

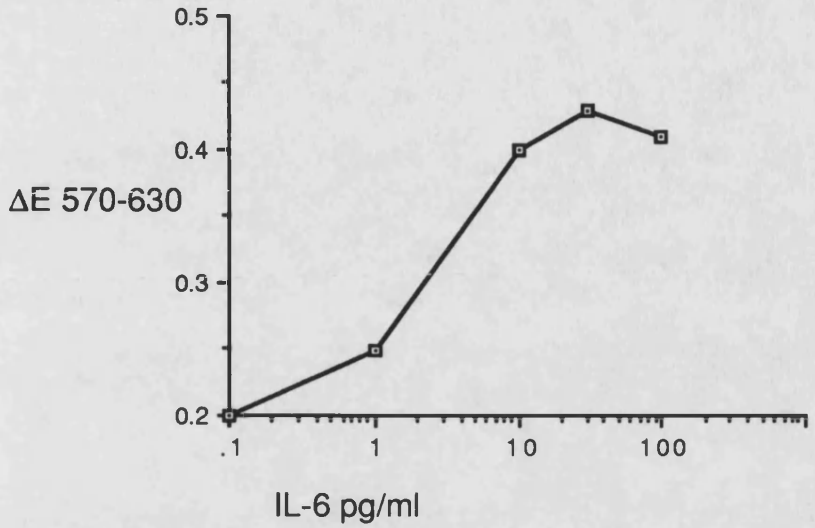
ROS 17/2.8 cells (described on page 113) were maintained in HAMS F10 medium, 10% (v/v) heat-inactivated FCS, 100 U/ml penicillin, 100 ug/ml streptomycin and 2 mM L-glutamine in 9 cm tissue culture dishes (HAMS F10 + 10% FCS). They were split at a ratio of 1:4 once or twice a week. The culture medium was removed from a confluent 9 cm tissue culture petri dish of ROS 17/2.8 cells, the cell layer washed once in PBS and trypsin-EDTA was added as described for human osteoblast-like cell passaging. After the cells were centrifuged at 1000 rpm for 7 minutes, the cell pellet was carefully resuspended in 4 ml of fresh HAMS F10 + 10% FCS and 1 ml added to a 9 cm tissue culture petri dish containing fresh HAMS F10 + 10% FCS. If the cells were to be used for experimental purposes, the pellet was resuspended and treated the same way as described for the human osteoblast-like cells.

IL-6 Bioassay

Principal

This bioassay utilizes the B9 murine hybridoma cell line which is an IL-6 dependant line and a sensitive variant of B13.29 cells (Helle et al 1988a). B9 cells proliferate according to the amount of IL-6 added and are not responsive to any other cytokine except for a slight stimulation with murine IL-4. The proliferation of B9 cells can be stimulated by as little as 1 pg/ml of IL-6 (see fig. 2.1). The specificity of this assay is described on page 110 (Chapter 4). Intra and inter assay variation was routinely less than 10%.

Fig. 2.1 Typical standard curve



Maintenance of the B9 cells

The B9 cells were maintained in RPMI supplemented with 5% (v/v) FCS, 100 U/ml penicillin, 100 ug/ml streptomycin, 2 mM L-glutamine $5x10^{-5}$ M 2-mercaptoethanol and 0.1% (v/v) monocyte conditioned medium (RPMI + 5% FCS). They were passaged when the cell density reached 1 million/ml of medium. 1 ml of medium containing cells was removed and passaged into 29 mls of RPMI + 5% FCS in a 75 cm^3 flask.

Method for bioassay

B9 cells were washed twice in RPMI + 5% FCS lacking IL-6. The cells were then counted using a haemocytometer and adjusted to a concentration of 50,000 cells/ml. An aliquot of 2-mercaptoethanol was then added to a final concentration of $5x10^{-5}$ M and 100ul of the cell suspension was dispensed into 96 well flat bottomed plates.

The conditioned media samples to be assayed were added to the wells. It was necessary to dilute the samples in order to obtain a reading on the linear part of the standard curve. 100ul of sample was then added to the 100 ul of B9 cells, thus resulting in a two fold dilution of the samples.

The standard curve was constructed using recombinant human IL-6 (rhIL-6) to give final concentrations of:- 100, 30, 10, 1, 0.1 and 0 pg/ml. Diluent medium which was used for the samples, was also used for the preparation of the standard curve. The plates were then incubated for 68 h after which 20 ul of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5mg/ml PBS) was added to each well for 4 h. The metabolically active B9 cells converted the MTT to formazan blue crystals. After 4 h, the reaction precipitate was solubilized in 50 ul of acidic (0.04M HCl) isopropanol after careful aspiration of the medium.

Once the precipitate was fully dissolved, the absorbance could be quantified on an ELISA platereader (Dynatech). The absorbances were measured at wavelengths of 570nm (test) and 630nm (reference). A standard curve was plotted on logarithamic paper and the IL-6 content of the samples determined from the curve.

Preparation of monocyte conditioned medium

Blood was collected from normal volunteers in heparinised tubes. The mononuclear fraction was isolated by diluting the blood with PBS (10 ml blood : 14 ml PBS) and layering carefully over Ficoll-hypaque (12 ml diluted blood : 5 ml Ficoll). This was then centrifuged at 1450 rpm for 30 minutes and the mononuclear fraction aspirated from the interface between the plasma and the Ficoll with the erythrocytes and neutrophils having been pelleted. The mononuclear cells were washed with RPMI + 5% (v/v) FCS and centrifuged at 1000 rpm for 7 minutes. The cell pellet was suspended in the 10 ml of the same medium and plated into a 9 cm tissue culture

petri dish. This was left overnight at 37°C and the following day the monocyte conditioned medium was removed and stored in aliquots at -20°C. This was used as a crude source of IL-6 and any other factors produced by these cells did not affect the B9 assay (Helle et al 1988b).

Preparation of probes

Preparation of competent bacteria

A 10 ml overnight culture of *E. Coli* (XL1 blue) bacteria was grown in LB medium (see Appendix 1) and the following day, 1 ml of this culture was transferred to 100 ml of LB medium and cultured for 2-4 hours to a cell density of 5 x 10^7 cells/ml (OD_{600}=1). Once the bacterial culture had reached the required optical density, they were chilled on ice for 10 minutes and centrifuged at 4000g for 5 minutes at 4°C. The supernatant was then discarded and 0.5 volumes of ice cold fresh, sterile 50 mM $CaCl_2$, 10 mM Tris HCl (pH 8) solution was added. This was placed on ice for 15 minutes and then centrifuged at 4000g for 5 minutes at 4°C. The supernatant was discarded and 0.066 volumes of the 50 mM $CaCl_2$, 10 mM Tris HCl (pH 8) solution was added to the cell pellet on ice. Aliquots (0.2 ml) were then dispensed into pre-chilled eppendorf tubes and stored at -70°C.

Transformation of bacteria

Competent XL 1 blue *E. coli* bacteria were removed from -70°C storage and thawed slowly on ice. The equivalent of 10 ng of the appropriate cDNA (cDNA enclosed within the vector DNA) was added to 50 ul of bacteria for 30 minutes on ice. The bacteria were then 'heat shocked' by first immersing them in a 42°C water bath for 2 minutes and subsequently transferring them onto ice for a further 2 minutes. The bacteria were allowed to recover by addition of 0.5 ml LB medium at 37°C for 30 minutes. The bacteria were then plated out on L-ampicillin (amp) plates (see Appendix 1) (25 mM stock used at a 1: 500 dilution). This was performed using a

sterile glass spreader under aseptic conditions. Once the bacteria were added, the plates were left inverted in the incubator overnight at 37°C. A control plate consisting of competent bacteria without DNA, was also prepared at the same time. The following day no colonies should be detected on the control amp plate since the bacteria do not contain resistance to ampicillin. A few colonies from the positive plate were then picked and grown up in 10 ml LB medium (containing amp) for 4 hours at 37°C. A small quantity of DNA could then be prepared to verify that the bacteria contained the cDNA/vector (see below).

Small scale plasmid preparation

For the small scale preparation of plasmid DNA, 1.5 mls of the bacteria were poured into an eppendorf tube and centrifuged (15,000 rpm) at room temperature for two minutes. The cell pellet could be enlarged by adding a further 1.5 mls of the bacterial culture and re-centrifugation. The remaining bacteria were kept at 4°C for subsequent large scale plasmid preparation. The cell pellets were sequentially resuspended in various solutions to isolate the plasmid DNA (see Appendix 1). The contents of the tubes were centrifuged at 15,000 rpm for 5 minutes, the supernatant was transferred to a clean tube and 2 x (v/v) absolute ethanol was added. This was allowed to stand for 30 minutes on ice to permit the DNA to precipitate. The pellet of precipitated DNA was washed in 100 ul of 70% ethanol and dried under vacuum. The DNA was then dissolved in 50 ul of 1 x TE (see Appendix 1) and quantified on the spectrophotometer (see Appendix 1).

The equivalent of 1 ug of DNA was digested with appropriate enzymes to excise the cDNA from the vector DNA. Enough enzyme was required to cut 1 ug of DNA per hour at 37°C (generally a slight excess of enzyme was used). 20 ug/ml of RNase A (RNA exonuclease) was also added to the digestion mixture. This treatment removed RNA from the preparation which would obscure the DNA bands on an

ethidium bromide-stained agarose gel when viewed over an ultra violet (UV) transilluminator. The digestion was allowed to proceed for 1.5 h at 37°C and 1 ul of DNA 'loading dye' (see Appendix 1) was subsequently added. The samples were electrophoresed on a 1% (w/v) agarose gel together with molecular weight markers and some undigested vector + insert DNA (see Appendix 1). The gel was then viewed over a UV transilluminator and the size of excised DNA assessed with the aid of λ DNA cut with Hind III and Eco RI used as the molecular weight markers. If the expected size of DNA was obtained, the remaining bacteria culture could then be used for large scale preparation of plasmid DNA.

Large scale plasmid preparation

About 5 mls of the bacteria were added to 250-500 mls LB medium containing amp (25 mM stock used at a 1: 500 dilution). This was cultured overnight in a shaking incubator at 37°C. The whole bacterial culture was subsequently centrifuged at 9000 rpm for 10 minutes. A 'Qiagen kit' was then used to purify the vector (containing insert) DNA.

The DNA was precipitated with 0.8 volumes of isopropanol and the pellet was dissolved in 50 ul of 1 x TE and quantified (see Appendix 1). 1 ug of plasmid DNA was digested as described previously in order to ascertain the presence of the cDNA/vector DNA. There was no necessity to perform an RNase digestion as the Quiagen kit contained an RNase digestion stage. The DNA was electrophoresed on a 1% (w/v) agarose gel as described previously and the remaining DNA was stored at -20°C.

Purification of the DNA

The equivalent of 5 ug of vector + insert DNA was digested with the two appropriate enzymes for excising the insert DNA from the vector DNA. If enzymes required

different buffers, the lower salt buffer enzyme was used first for 1.5 h at 37°C. The salt concentration was then altered for the second enzyme and the restriction digest continued for a further 1.5 h at 37°C (unless the enzyme required an alternative temperature).

One tenth of the digest was removed and checked on an agarose gel, as described previously, to assess whether the digestion had been completed. If this was satisfactory, the remaining DNA was run out on a 1% (w/v) agarose gel and the appropriate ethidium bromide-stained DNA band was excised from the gel over a UV transilluminator. A kit was used to purify the DNA (Gene Clean kit) using glass milk silica particles which bind to the DNA. The DNA was subsequently eluted twice at 50°C with 10 ul of 1 x TE. An aliquot (2 ul) of the purified insert cDNA was removed and assessed on another gel for recovery of the DNA. The remaining purified DNA was stored at -20°C until use for hybridisation.

Statistics

All statistical differences were determined using the Student's *t* test. $P < 0.001$***, $p < 0.01$**, $p < 0.05$*.

Where the term 'representative experiment (n=x) is written under the figure legends, this means that 'x' experiments were performed and a representative experiment was chosen. Each treatment was performed in triplicate. The errors represent the variation between the triplicate wells.

In Chapter 5, the original magnification is shown on the bottom right hand side of the photographs. Where the same magnification was used for all the photographs within the figure, the magnification is only shown once.

APPENDIX 1

Preparation of competent bacteria

Using a sterile loop; the bacteria were transferred from a frozen vial stored in liquid nitrogen at -70°C to a 10 ml culture of LB medium containing ampicillin (25 mM stock used at a 1: 500 dilution).

LB medium

bacto-tryptone 10g

bacto-yeast extract 5g

NaCl 10 g

The reagents were dissolved in 800 ml deionised water, the pH adjusted to 7.5 with NaOH and the volume made to 1 litre. This was then autoclaved immediately.

Preparation of L ampicillin plates

These were prepared if the vector contained an ampicillin resistance region.

LB medium was made up as above and 1.5% (w/v) agar was added. This was heated thoroughly until all the agar dissolved and the solution was cooled to about 50°C before addition of ampicillin (50 ug/ ml). The plates were poured under aseptic conditions and allowed to set. They were then transferred to a 37°C incubator and inverted with the lids off in order to dry off the condensation.

Small scale preparation of plasmid DNA

Isolation of the vector + insert DNA

100 ul of solution 1 (50 mM glucose, 25 mM Tris, 10 mM EDTA pH 8 and freshly added lysozyme 2 mg/ml) was added to the bacterial pellet. Following this, 200 ul of solution 2 (0.2 M NaOH, 1% SDS) and 150 ul of cold solution 3 was added (5 M potassium acetate, pH 4.8 with glacial acetic acid). Contents

were gently mixed in between each addition and the tubes were placed on ice for 5 minutes followed by centrifugation (15,000 rpm) for 5 minutes in a microfuge. The clear supernatant was carefully removed and the DNA precipitated by addition of 2 x the volume of absolute ethanol.

10 x TE pH 7.4

100 mM Tris pH 7.4

10 mM EDTA pH 8

These reagents were dissolved in distilled water (For pH 8 TE; Tris is made to pH 8).

Quantification of DNA

An aliquot of DNA (usually 2.5 or 5 ul) was diluted in water to a total volume of 250 ul.

The absorbance was read in a silica cuvette at 260 nm and 280 nm and the ratio of absorbance at 260 nm : 280 nm was determined. A pure DNA preparation should have a ratio of 1.8 at 260 nm : 280 nm.

Concentration of DNA was calculated by:-

$$A_{260} \times 50 \times \text{dilution} = \text{ug/ml DNA}$$

(50 ug/ml of pure DNA has an OD of 1)

10X DNA 'loading dye'

0.25% bromophenol blue

0.25% xylene cyanol

25% ficol

Reagents were dissolved in distilled water.

The solution contained ficol, a heavy sugar which helped to sink the DNA to the bottom of the wells in the gel. 1 ul of loading dye was added to 9 ul of DNA sample (which was usually diluted in distilled water).

Agarose gel

For a 1% agarose gel, 1g of agarose was dissolved in 100 ml of 1 x tris acetate by boiling. 0.05% v/v ethidium bromide was added on partial cooling, the liquid poured into a gel mould containing a comb to form wells and allowed to set. The gel was placed in a tank containing 1 x tris acetate running buffer, the comb was removed and samples containing the dye were loaded. The samples were electrophoresed until the dye markers had migrated over half the length of the gel. In order to obtain a good resolution, the samples were often electrophoresed until the dye had migrated to the end of the gel. As DNA possesses a negative charge; the samples migrated towards the anode.

10 x tris acetate

0.4 M Tris

0.05 M sodium acetate

0.01 M EDTA

The reagents were dissolved in distilled water and the pH was adjusted to pH 8.1 with glacial acetic acid.

CHAPTER 3

THE EFFECTS OF EXOGENOUS IL-6 ON HUMAN AND RAT OSTEOBLAST-LIKE CELLS.

ABSTRACT

Interleukin 6 (IL-6) exerts well established effects on cells of the immune system as well as on a variety of other cell types. Many of its activities overlap with the well characterised cytokine IL-1 which is a potent agent in the control of bone turnover. Elevated levels of IL-1 and IL-6 in synovial fluid and serum have been implicated in the pathogenesis of inflammatory conditions such as rheumatoid arthritis. Several groups have studied the effects of IL-6 on bone resorption by osteoclasts, however, little work with respect to the actions of IL-6 on osteoblasts has been reported. The effects of recombinant human IL-6 (rhIL-6) on human osteoblast-like cells derived from explants of trabecular bone were investigated and compared with those exerted on ROS 17/2.8 cells. rhIL-6 used over the concentration range of 0.05 - 5 ng/ml had no observable effects on the cell proliferation, alkaline phosphatase activity or release of prostaglandins by either osteoblast-like cell model system. In addition, the synthesis of osteocalcin, IL-1 and TNF were not regulated by rhIL-6 in human osteoblast-like cells. Thus, from the parameters investigated in this study, IL-6 does not appear to be involved in the regulation of osteoblast activity. In addition, rhIL-6 does not exhibit similarities with IL-1 on the above osteoblast functions, nor does it modulate several IL-1-stimulated activities.

INTRODUCTION

IL-6 although distinct in structure from IL-1, exhibits strikingly similar activities such as stimulation of the synthesis of the acute phase proteins from human hepatocytes (Gauldie et al 1987, Castell et al 1989), induction of fever (Helle et al 1988a) and enhanced thymocyte proliferation (Lotz et al 1988). In addition, both cytokines stimulate the production of ACTH from rat anterior pituitary cells (Besedovsky et al

1986, Naitoh et al 1988) and are involved in hematopoiesis (Ikebuchi et al 1987). Both IL-1 and IL-6 are able to synergise with IL-3 to increase hematopoietic stem cell formation (Koike et al 1988, Hoang et al 1988, Wong et al 1988), although IL-1 was reported to be less effective than IL-6 (Koike et al 1988, Ogawa and Clark 1988). IL-1 has previously been shown to exert complex effects on bone metabolism. IL-1 is a potent bone resorptive agent (Gowen et al 1983, Dewhirst et al 1987, Stashenko et al 1987a, Sabatini et al 1988, Sato et al 1989) and induces osteoclast-like cell formation in long-term bone marrow culture (Pfeilschifter et al 1989, Kurihara et al 1991). IL-1 appears to exert variable effects on bone formation and matrix synthesis according to the culture conditions. Stashenko et al (1987b) observed that IL-1 inhibits matrix synthesis, whereas Canalis (1986) demonstrated that under certain conditions, the production of collagen type I in fetal rat calvarial osteoblast-like cells could be induced by IL-1. IL-1 also stimulates osteoblast proliferation (Gowen et al 1985, Ikeda et al 1988, Gowen 1988, Evans et al 1989b, Evans et al 1990b, Rickard et al 1990) which may lead to subsequent bone formation (Boyce et al 1989) related to the successful coupling with previous bone resorption. In contrast, the activities of IL-6 are not so well established with respect to the bone remodelling cycle. The potential involvement of IL-6 in bone metabolism is supported by a number of lines of evidence. IL-6 is present in the synovial fluid in patients with inflammatory arthropathies such as rheumatoid arthritis in contrast to patients suffering from osteoarthritis (Hirano et al 1988, Guerne et al 1989). IL-6 is likely to be involved in the disease multiple myeloma (Kawano et al 1988, Asaoku et al 1988, Bataille et al 1991) which, like rheumatoid arthritis, is associated with extensive bone resorption (Mundy et al 1974). There are also reports of a bone resorbing effect of this cytokine in murine and rat bone models (Lowik et al 1989, Black et al 1990, Suda et al 1990). In contrast, others have been unable to show any stimulatory effects of IL-6 on bone resorbing cells (Al-Humidan et al 1991, Barton et al 1990).

There are relatively few reports of the effect of IL-6 on osteoblast activity however, and since IL-1 and IL-6 share certain similar activities discussed earlier in this introduction,

a comparison of the effects of these cytokines were studied on human osteoblast-like cells. IL-1 has previously been demonstrated to exert a number of effects on osteoblast-like cells including the stimulation of prostaglandin production, cell proliferation (Gowen 1988, Evans et al 1989b, Evans et al 1990b) and the induction of other cytokines such as TNF (Gowen et al 1990). Therefore, the effects of IL-6 on cell proliferation, production of prostaglandin E_2 and TNF in the presence and absence of IL-1 was examined. In addition, the effects of IL-6 on the production of IL-1 were also studied. Alkaline phosphatase and osteocalcin expression are markers for the maturing osteoblast phenotype and so the ability of IL-6 to modulate the activity of these enzymes was also investigated.

METHODS

TNF bioassay

Principle

The murine fibrosarcoma WEHI 164 clone 13 cell line (Espevik and Nissen-Mayer 1986) is lysed in the presence of TNFα and TNFβ. As a consequence an inverse relationship exists between cell number and TNF concentration. This therefore represents a cytotoxic bioassay.

Maintenance of WEHI cells

The WEHI 164 clone 13 cells were cultured in RPMI 1640 containing 10% (v/v) FCS, 100 U/ml penicillin, 100 ug/ml streptomycin and 2mM L-glutamine (RPMI 1640 + 10% FCS). Cells were passaged at, or before, confluency. This cell line is not cell contact inhibited, and so cell viability and attachment is reduced on prolonged culture. For the purpose of cell passaging, the cells were trypsinised for 3-5 mins. Detached cells were removed and centrifuged (7 minutes, 1000 rpm, 22°C) and resuspended in 10 ml of medium. New cell cultures were established in culture flasks using a 1:10 split ratio. Cells were adherent 4 h post-passage.

Method

Following trypsinisation and centrifugation, the cells were resuspended in 10 ml RPMI 1640 + 10% FCS and the cell number determined. Cells were then passaged into flat-bottomed 96-well plates at a density of $2x10^4$ cells per well in a total media volume of 100 ul. Cells were allowed to adhere to the tissue culture well following a 3-4 h incubation period at 37°C.

100 ul aliquots of sample, such as bone cell conditioned medium, (giving a 2-fold dilution of the samples) and 100 ul of rhTNFα standards made at 2x the final concentration were then added. A TNFα standard curve was constructed in triplicate over the concentration range of 0, 0.001, 0.01, 0.1, 1.0, 10, 100, 1000 U/ml. The WEHI 164 clone 13 cells were incubated with the samples at 37°C for a total period of 24 h. After 20 h, 10 ul of MTT solution (5 mg/ml in PBS) was added to each well and incubated for the final 4 h. Viable cells metabolized the tetrazolium salt to a blue/purple coloured formazan metabolic product. The medium was then aspirated and 50 ul isopropanol containing 0.04 M HCl was added which solubilised the formazan blue crystals and bleached any residual medium which would interfere with the absorbance. The absorbance was read on an ELISA plate reader using reference and test wavelengths of 630 nm and 570 nm respectively. The % cytotoxicity was calculated using the formula:-

$$1 - \frac{\text{(OD sample)}}{\text{(OD zero standard)}} \times 100\%$$

A standard curve of % cytotoxicity against TNF U/ml was plotted. The % cytotoxicity for each sample was then calculated and the TNF concentration was determined using the standard curve. Intra and inter assay variation was routinely less than 10%.

IL-1 bioassay

Principle

The D10N_4M murine T cell line (Hopkins and Humphreys 1989) proliferates in response to IL-1 and, to a lesser extent, with IL-2 and murine IL-4. The assay is therefore performed in the presence of an excess concentration of IL-2 to saturate the effects of any IL-2 that may be present in the test samples.

Maintenance of cells

Cells were maintained in RPMI 1640 containing 10% (v/v) FCS, 100 U/ml penicillin, 100 ug/ml streptomycin, 2 mM L-glutamine, rhIL-1α (2-4 U/ml), rhIL-2 (10-30 U/ml), concanavalin A (5 ug/ml) and 2-mercaptoethanol ($5x10^{-5}$ M). Under optimal growing conditions, the cells formed large clumps usually visible to the naked eye. For general maintenance the cells were split 1:10 twice weekly by transferring 2 ml of the cell suspension into a new flask containing 18 ml of medium with the added growth factors.

Method

Cells were pelleted by centrifugation (1000 rpm, 7 minutes, 22°C) and resuspended in RPMI 1640 containing 10% (v/v) FCS and re-centrifuged to remove residual traces of growth factors. Since the assay is performed in the presence of concanavalin A and saturating concentrations of IL-2, the pellet was then resuspended in RPMI 1640 containing 10% (v/v) FCS, 20-40 U/ml of IL-2 and 6-10 ug/ml of concanavalin A. A 100 ul aliquot of cells was plated into a 96-well round-bottomed plate at a density of $1x10^4$ cells per well and 100ul of conditioned medium was added. This is equivalent to a 2-fold dilution of the sample and of the medium containing the cells and factors. A standard curve was constructed using rhIL-1α as the standard in triplicate. A 100 ul aliquot of rhIL-1α was added to the appropriate wells to give a final concentration range of 0, 0.001, 0.003, 0.01, 0.03, 0.1, 1.0, and 10 U/ml. The cells were incubated for 72 h at 37°C and pulsed with 1 uCi/well of 6-[^{3}H]-thymidine for the final 4 h. A Skatron cell harvester was used to collect the radioactivity incorporated into the cells. This radioactivity was retained on paper discs (Titertek) which were allowed to dry and

placed in scintillation vials containing 0.5 ml of Optiscint scintillation liquid (LKB). Incorporated radioactivity was assessed by liquid scintillation counting using a β-counter. A standard curve plot of 6-[^{3}H]-thymidine incorporation (cpm) against IL-1 concentration (U/ml) was constructed from which the IL-1 concentration of the samples was extrapolated.

Cell proliferation

Method

To assess the effect of various agents on the proliferation of the human osteoblast-like cells and ROS 17/2.8 cells, 10,000 cells/well were passaged into a 48-well plate for a 24 h period, prior to a 24, 48 or 72 h incubation in the appropriate medium containing 3% or 10% (v/v) FCS, or under serum-free conditions, together with the test agents. The cells were incubated with 1uCi 6-[^{3}H]thymidine for the final 6 h of the experiment. The experiment was terminated by the removal of the medium and the cell layer was washed twice with PBS. Trypsin-EDTA (100 ul) solution was added to each well and incubated until the cell layer was detached. Medium (200 ul) containing 10% (v/v) FCS was added to each well and the contents were transferred to LP4 tubes on ice. Each well was rinsed again with 200 ul of the same medium and the washings transferred to the corresponding LP4 tubes. The DNA was precipitated by addition of 50 ul of 60% (w/v) trichloroacetic acid (TCA) and the contents of the tubes were vortexed and placed at 4°C for 20 h.

The samples were centrifuged at 2000 rpm for 10 mins at 4°C and the supernatant containing the unincorporated 6-[^{3}H]thymidine was discarded. The precipitate was dissolved in 1 ml warm dH_2O and vortexed. Once dissolved, the tubes were placed on ice and the DNA was reprecipitated with 100 ul of 60% (w/v) TCA. The tubes were placed at 4°C for 3-5 h and then centrifuged as before. The supernatant was discarded and the precipitate was vortexed and dissolved in 0.5 ml warm dH_2O. In some cases where the final precipitate would not dissolve in dH_2O, a small amount of NaOH (3 M) was added to aid this process.

The contents of each LP4 tube were then transferred to scintillation vials and 4 ml of (aqueous) scintillation fluid (LKB Optiphase 'Hisafe'3) was added to each vial. Incorporated 6-[^{3}H]thymidine was determined by counting for 60 seconds on a ß-counter.

Alkaline phosphatase assay and protein estimation

Alkaline phosphatase

Alkaline phosphatase is the generic name given to a group of phosphatases which display maximum activity in the range pH 9.0-10.5. They are hydrolases and an alternative name is orthophosphoric monoester phosphohydrolase. The reaction that these enzymes catalyse can be represented:-

$$ROP + H_2O \Rightarrow ROH + POH$$

Phosphoester Alcohol + Orthophosphate

In the assay described above, 4-nitrophenylphosphate is the substrate and p-nitrophenol is the reaction product.

Method

Human bone cells or ROS 17/2.8 cells were plated out in the appropriate medium in 24-well plates at a cell density of 40,000 cells/well and allowed to attach to the tissue culture substratum for 24 h. The medium was replaced with new EMEM or HAMS-F10 containing 10% (v/v) FCS or serum-free, together with the test agents, for a 24, 48 or 72 h incubation period. After this time the medium was removed and either stored for bioassays or discarded. The plates were placed on ice and 200 ul of 0.1% (v/v) Tween in ddH_2O added to each well. The cell layers were scraped thoroughly and the contents of each well transferred to an LP3 tube. For the determination of alkaline phosphatase activity, 50 ul of each sample or p-nitrophenol standard (see Appendix 2) was added to an LP3 tube in duplicate. A 100 ul aliquot of the remaining

sample was transferred to another LP3 tube and stored at -20°C for protein estimation - see Protein Estimation. To 100 ml of assay buffer (see Appendix 2) 46.4mg 4-nitrophenylphosphate was added immediately prior to use. To each sample and standard, 500 ul of this solution was added and incubated at 37°C for 30-60 minutes until optimal colour development was obtained. The reaction was stopped by adding 1 ml of 0.2 M NaOH to each tube.

The absorbance was measured at 410 nm using a spectrophotometer or by transferring a 300 ul aliquot into a 96-well plate and using an ELISA platereader (Dynatech). For the standard curve, the absorbance was plotted against p-nitrophenol concentration. Hence the amount of p-nitrophenol generated in the samples could then be determined by extrapolation.

Protein Estimation

This method is based on the Folin phenol reagent of Folin and Ciocalteu, the active constituent of which is phosphomolybdic-tungstic mixed acid. The mixed acids in the preparation are the final chromagen and involve the following chemical species:

$3H_2O.P_2O_5.13WO_3.5MoO_3.10H_2O$

and $3H_2O.P_2O_5.14WO_3.4MoO_3.10H_2O$

Proteins affect the reduction of the mixed acid by loss of 1, 2, or 3 oxygen atoms from tungstate and/or molybdate, thereby producing one or more of several possible reduced species which have a characteristic blue colour (λ_{max} 745-750 nm, λ_{min} 405 nm). Copper is thought to chelate with the peptide structure and facilitate electron transfer to the mixed acid chromagen, particularly in the vicinity of amino acid functional groups, thereby increasing the sensitivity to protein.

Method

A 100 ul aliquot of the scraped cell layer in 0.1% (v/v) Tween as described for alkaline phosphatase was used to determine the amount of cellular protein present in the well. The 100 ul aliquot was placed in an LP3 tube and a standard curve constructed using

BSA at various dilutions (see Appendix 2). 500 ul of alkaline copper reagent (see Appendix 2) was added and the contents of the tubes vortexed and left to stand for 10 minutes. After this time, 50 ul of freshly diluted Folin's Reagent (1:2 in ddH_2O) was added to each tube and the contents were mixed immediately and left to stand for 30 minutes. A 300 ul aliquot of sample/standard was transferred to a 96-multiwell plate and the absorbance determined using an ELISA plate-reader at a wavelengh of 750 nm. A graph of absorbance against protein concentration was then plotted and the amount of protein in the samples determined.

The alkaline phosphatase activity was expressed as umol p-nitrophenol released/ug protein/h.

Osteocalcin assay

Principle of assay

Osteocalcin concentration was determined using a competitive RIA. Unlabelled osteocalcin released into the culture media and radiolabelled ^{125}I-osteocalcin compete in binding to the primary osteocalcin antibody. The secondary antibody then binds to the osteocalcin/primary antibody complex to form an immunoprecipitate which is collected at the end of the assay.

When more osteocalcin is released by the cells into the supernatent, less ^{125}I-osteocalcin is able to bind to the primary antibody and so the associated radioactivity in the precipitate is decreased. In contrast, if less osteocalcin is released into the culture supernatent, more ^{125}I-osteocalcin is able to bind to the primary antibody and so the radioactive counts increase.

Method

Conditioned medium, either saved from the alkaline phosphatase assay or prepared freshly by plating out human bone cells as for the alkaline phosphatase assay, was dispensed into LP4 tubes in 50 ul aliquots in triplicate. A standard curve of 100, 50,

33, 20, 10, 5, 3.3, 2, and 1 ng/ml was constructed using bovine osteocalcin standard (Biogenesis) diluted in assay buffer (see Appendix 2).

Tubes were also prepared to assess total counts (TC) (3 LP4 tubes x 50ul ^{125}I-osteocalcin), non specific binding (NSB) (3 LP4 tubes x 50ul non-immune rabbit serum diluted to 2.5% (v/v) in assay buffer) and maximum binding (MB) (3 LP4 tubes). Bovine osteocalcin (Biogenesis) was radioiodinated using the Chloramine-T method (prepared by Dr. D.B. Evans).

Assay buffer, then primary antibody followed by ^{125}I-osteocalcin were added to the tubes (see Appendix 2). After all of the additions, the contents of the tubes were vortexed and incubated for 24 h at 4°C. After 24 h, 50 ul (0.5 U) of the secondary antibody (Goat anti-rabbit IgG, Calbiochem) was added to all tubes except total counts. The contents of the tubes were vortexed and incubated overnight at 4°C again. After this time, the tubes were centrifuged at 3000 rpm for 25 minutes at 10°C and the supernatant carefully removed by aspiration. The radioactivity of the immunoprecipitate was determined in a γ-counter and the osteocalcin in ng/ml calculated from the standard curve by on-line computer.

Prostaglandin assay (PGE_2)

Principle

Radiolabelled and unlabelled PGE_2 compete for the binding site on a specific PGE_2 antibody. Following incubation, the unbound radiolabelled PGE_2 is then removed by treatment with Dextran-coated charcoal. The antibody-radiolabelled PGE_2 complex remaining in the supernatent is then counted on a β-counter.

Method

Human bone cells or ROS 17/2.8 cells were plated out as described for the cell proliferation experiments and allowed to adhere for 24 h prior to the addition of the test agents in the appropriate medium in the presence and absence of FCS. The bone cell-conditioned medium was removed after 24, 48 or 72 h and stored for PGE_2 assay.

An aliquot of appropriate medium (not incubated with bone cells) was saved for constructing the PGE_2 standard curve.

Aliquots of the conditioned medium (100 ul) were transferred into LP4 tubes on ice and assayed in duplicate. The standard curve (100 ul aliquots assayed in triplicate) was made by diluting stock PGE_2 standard (see Appendix 2) to 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.039, 0.020 and 0.010 ng/ml in non-conditioned medium. Tubes were also prepared to assess total counts, non-specific binding and maximum binding (0 standard) and placed on ice. Assay buffer (see Appendix 2), 100 ul [5,6,8,11,12,14,15 (n) ^3H-PGE_2] (160 Ci/mmol) (diluted 1:15,000 in assay buffer) and finally 100 ul antibody (one vial of anti-PGE_2 (Steranti Research) was dissolved in 100 ml assay buffer) were added to the tubes. The contents of the tubes were vortexed and placed at 4°C overnight. The following day, a charcoal/dextran mixture (300 mg Norit A charcoal + 62.5 mg Dextran T70 in 100 ml working buffer) was prepared and mixed on ice for 30 minutes prior to use. A 200 ul aliquot of this mixture was dispensed into all the LP4 tubes except for the total counts. The contents of the tubes were subsequently vortexed and left for 5 minutes. Following this, the tubes were centrifuged at 4°C for 15 minutes at 3000 rpm. A 200 ul aliquot of the supernatent was transferred into 4 ml of Optiphase aqueous scintillant, vortexed and counted on a liquid scintillation β counter.

Calculation:- $$\frac{\text{Sample count per minute - NSB}}{\text{Total counts}} \times 100 = \%\ \text{Binding}$$

A graph of % binding against PGE_2 concentration was plotted and from the % binding of samples the amount of PGE_2 in the conditioned medium was determined.

RESULTS

TNF production

Wherever possible, the studies of the effects of IL-6 on osteoblasts were undertaken in serum-free culture in order to exclude the possibility of interactions with many other cytokines and growth factors contained within FCS. This presented difficulties when studying the production of TNF and IL-1 by human trabecular bone cells, since bioassayable levels of TNF and IL-1 were not readily detectable under serum-free conditions even when a known positive stimulator was present. Therefore these experiments had to be conducted in media containing 10% (v/v) FCS. Indomethacin is a non-steroidal anti-inflammatory drug known to inhibit cyclooxygenase activity (Flower 1974) and hence prostaglandin synthesis (Vane 1971). This drug was added to abolish prostaglandin synthesis since prostaglandins are known to modify certain actions of cytokines on cell proliferation (Gowen et al 1985) and cytokine production (Kunkel and Chensue 1985).

After a 6 h culture period, in 10% (v/v) FCS-containing EMEM with indomethacin (1.4 uM), unstimulated human osteoblast-like cells produced a small amount of bioactive TNF (fig. 3.1). Over a series of 10 experiments in medium containing 10% (v/v) FCS, the basal levels of TNF ranged from <0.001 - 0.302 U/ml TNF. rhIL-1α (10^{-13} - 10^{-11} M) consistently stimulated TNF release in human bone cells, although the levels of induction varied from one patient to another over the range of 0.62 - 5.7 U/ml. rhIL-6 over the concentration range 0.05 to 5 ng/ml did not affect basal or rhIL-1α-induced release of TNF (fig. 3.1). ROS 17/2.8 cells did not produce detectable quantities of TNF in 10% (v/v) FCS-containing HAMS F10 and 1.4 uM indomethacin (data not shown).

IL-1 production

Unstimulated levels of IL-1 in the conditioned medium of human osteoblast-like cells were very low (<0.001 U/ml). In the presence of indomethacin (1.4 uM), and 10% (v/v) FCS-containing EMEM, human osteoblast-like cells released 0.03 U/ml IL-1

when stimulated with rhTNFα (10^{-7} M) for 24 h and this was therefore used as a positive stimulatory control. Basal levels were unaffected by rhIL-6 over the dose range of 0.05 - 5 ng/ml (fig. 3.2). Non-conditioned media containing the test agents were added at least once directly to the TNF and IL-1 bioassays in order to confirm that the effects measured were due to agents released into the medium by the osteoblast-like cells and were not due to the interference with the test agents themselves (data not shown).

PGE2 production

A 5-7 fold increase in basal PGE_2 levels was observed with 10^{-12} M rhIL-1α (Fig. 3.3) after a 24 h culture period under serum-free conditions. After 48 and 72 h of serum-free culture, the stimulatory effect of rhIL-1α was still evident (fig. 3.3). rhIL-6 over the concentration range 0.05 - 5 ng/ml alone exerted no modulatory effect on basal PGE_2 production at any time point studied or following stimulation with 10^{-12} M rhIL-1α (fig. 3.3).

rhIL-6 (0.05 - 5 ng/ml) did not modulate the basal or rhIL-1α-stimulated (10^{-12} M) PGE_2 production in ROS 17/2.8 cells after incubation for 72 h in serum-free conditions (fig. 3.4). It was of interest to note that the basal and rhIL-1α-stimulated PGE_2 production from these osteosarcoma cells was approximately 100-fold lower than levels produced by human osteoblast-like cells. The basal production of PGE_2 by ROS 17/2.8 cells after a 72 h serum-free culture was 0.05 ng/well compared with 4ng/well in human osteoblast-like cells. rhIL-1α-stimulated PGE_2 levels under the same cultural conditions varied from 0.09 ng/well in the ROS 17/2.8 cells to 22 ng/well in the human trabecular bone cells.

Cell proliferation

rhIL-6 (0.05 - 5 ng/ml) in the presence of indomethacin (1.4 uM), did not modulate the cell proliferation of the human osteoblast-like cells in serum-free medium when incubated for 24, 48 or 72 h (Fig. 3.5). IL-1 has been previously demonstrated to

stimulate the cell proliferation of the human osteoblast-like cells, thus rhIL-1α was used as a positive stimulatory control (Gowen 1988, Evans et al 1989b, Evans et al 1990b). In contrast to these published observations, IL-1 did not stimulate cell proliferation in serum-free culture in the present studies.

The effect of indomethacin (1.4 uM) on the basal cell proliferation in the presence and absence of IL-6 (0.05 - 5 ng/ml) was studied in human trabecular bone cells (Table 3.1). Indomethacin did not influence cell proliferation under these conditions in either serum-free or 3% (v/v) FCS-containing medium (Table 3.1). IL-6 had no effect on cell proliferation under any of the conditions used. However, addition of serum (3% v/v) did cause an increase in the basal proliferation of the human osteoblast-like cells.

rhIL-6 (0.05 - 5 ng/ml) was also ineffective when studying the proliferation of ROS 17/2.8 cells under serum-free conditions in the presence of indomethacin (1.4 uM) after 24, 48 and 72 h incubation (Fig 3.6). In contrast, rhIL-1α (10^{-11} M) stimulated the proliferation of the ROS 17/2.8 cells after a 48 and 72 h incubation. This response was unaltered by the simultaneous addition of rhIL-6 (Fig. 3.6).

Alkaline phosphatase expression

$1,25(OH)_2D_3$ stimulates the expression of alkaline phosphatase in human trabecular bone cells (Beresford et al 1986, Evans et al 1990a) and ROS 17/2.8 cells (Majeska and Rodan 1982). The action of rhIL-6 was examined to determine whether it exhibited any regulatory action on basal and $1,25(OH)_2D_3$-stimulated alkaline phosphatase production in human bone cells cultured in the presence and absence of serum. rhIL-6 (0.05 - 5 ng/ml) exerted no effects on both the unstimulated and $1,25(OH)_2D_3$-stimulated (10^{-8}M) levels of alkaline phosphatase in the presence (fig. 3.7) or absence (fig. 3.8) of serum after a 72 h culture period.

ROS 17/2.8 cells expressed high basal alkaline phosphatase levels which were only slightly, but consistently, stimulated with $1,25(OH)_2D_3$ (10^{-8}M). rhIL-6

(0.05 - 5 ng/ml) did not modulate the basal or $1,25(OH)_2D_3$ (10^{-8}M)-stimulated alkaline phosphatase expression after a 72 h incubation in serum-free culture (fig. 3.9).

Osteocalcin expression

The production of osteocalcin by osteoblasts is stimulated by $1,25(OH)_2D_3$ (Beresford et al 1984b, Evans et al 1990a). rhIL-6 (0.05 - 5 ng/ml) did not affect the basal or $1,25(OH)_2D_3$-stimulated production of this bone matrix protein after a 72 h serum-free culture period (fig. 3.10).

DISCUSSION

These studies were devised to determine whether the actions of IL-6 were similar to that of IL-1 on osteoblast-like cells and whether IL-6 modulated any of the IL-1 responses. There are definite differences between the actions of IL-1 and IL-6 observed on bone cell function in contrast to the similarities observed in the immune system. Using two osteoblast-like cell models IL-6, unlike IL-1, did not affect the basal expression of any of the parameters studied. Furthermore, IL-6 (0.05 - 5 ng/ml) did not appear to alter the phenotype of the osteoblast-like cells with respect to alkaline phosphatase and osteocalcin levels. In addition, IL-6 did not modulate any of the effects of IL-1 on the cell proliferation, cytokine and prostaglandin E_2 production by either osteoblast-like model system.

Other groups have not studied the effects of IL-6 on cytokine production in osteoblast-like cells. However, Kurihara et al (1990) demonstrated that IL-6 increased the formation of osteoclasts in long-term human bone marrow culture by inducing the expression of IL-1. By the use of a specific ELISA, they were able to demonstrate an increased production of IL-1 following addition of IL-6, although the IL-6-responsive cells within the marrow culture were not determined. IL-6 is reported to induce IL-1 secretion in fibroblasts, marrow stromal cells and glial cell lines (Kishimoto and Hirano 1988). It is possible that some cells within the bone microenvironment are capable of

being induced by IL-6 to release IL-1, although studies presented in this chapter would indicate that osteoblasts are not the primary target for IL-6.

IL-6 did not modulate either the basal or IL-1α-stimulated PGE_2 production in human osteoblast-like cells or ROS 17/2.8 cells. Bunning et al (1990) also demonstrated that IL-6 did not regulate PGE_2 expression in human articular chondrocytes. Contrasting studies from Fang and Hahn (1991) using UMR 106-01 rat osteoblast-like cells, demonstrated that IL-6 was capable of stimulating PGE_2 production. Their results were somewhat unusual however, as indomethacin did not inhibit prostaglandin production.

Proliferation of human trabecular bone cells was not stimulated by IL-6 or IL-1. The lack of effect of IL-6 could not be determined conclusively, as the positive control was also ineffective. There is some controversy as to whether IL-1 stimulates (Gowen et al 1985, Gowen 1988, Evans et al 1989b, Evans et al 1990b Canalis 1986) or inhibits the proliferation of osteoblast-like cells (Stashenko et al 1987b). The major arguement posed by Stashenko et al (1987b) was that the cultures in which cell proliferation was observed were heterogeneous, and therefore non-osteoblastic cells such as fibroblasts may have been proliferating in preference to the osteoblastic populations. This has been clarified in part by a new technique assessing cell proliferation on an individual cell basis (Rickard et al 1990). In these studies the osteoblast-like cells were induced to proliferate with IL-1 in the presence of [^{3}H]-thymidine. The cells were then subsequently induced to differentiate with $1,25(OH)_2D_3$, to a more mature phenotype as assessed by alkaline phosphatase expression. By use of this dual autoradiography-histochemical localisation method, cells which had been induced to replicate in response to IL-1 and then to subsequently differentiate into osteoblasts were specifically identified (Rickard et al 1990). It is likely that the lack of effects of IL-1 in the present study may have been due to either culture conditions (serum-free culture) or differences in donor responsiveness. Previously, the studies describing a stimulatory effect of IL-1 on human osteoblast-like cells were conducted in the presence of serum (Gowen et al 1985, Gowen 1988, Evans et al 1989b, Evans et al 1990b, Rickard et al 1990).

Under such conditions it is likely that important interactions between IL-1 and serum factors may have contributed to the proliferative action described for IL-1. However, an indication that IL-6 does not exert any proliferative action on human osteoblast-like cells is clearly demonstrated in the final chapter of this thesis.

In contrast to the human cells, IL-1 induced the cell proliferation of ROS 17/2.8 cells, which are highly proliferative osteosarcoma cells, and which were probably not adversely affected in the short term by serum-free culture. IL-6 however, did not modulate either the basal or IL-1-stimulated proliferation of the ROS 17/2.8 cells. In contrast, IL-6 increased the cell proliferation of UMR-106-01 cells (Fang and Hahn 1991). This occurred via a prostaglandin-dependent mechanism, associated with the endogenous stimulation of PGE_2 production by IL-6. The maximal effects observed with IL-6 occurred during the logarithmic growth phase of these UMR 106-01 cells. In the present studies, both the human and rat osteoblast-like cells were seeded at subconfluent levels as these were shown to be the most effective conditions for the induction of cell proliferation by IL-1 (Gowen 1988). IL-6 does influence the proliferation of other cell types however. This cytokine increases the proliferation of murine hybridoma cells (Helle et al 1988a, Helle et al 1988b), thymocytes (Helle et al 1988b) and AIDS-Kaposi sarcoma-derived cells (Miles et al 1990). Grossman et al (1989) demonstrated that IL-6 stimulates the proliferation of keratinocytes which may contribute to the clinical conditions associated with psoriasis. Others have demonstrated the inhibition of cell proliferation by IL-6 in mammary carcinoma cell lines (Tamm et al 1989, Tamm et al 1991).

The production of alkaline phosphatase and osteocalcin following stimulation by $1,25(OH)_2D_3$ in human osteoblasts is strongly inhibited by IL-1 and TNF (Gowen 1984, Gowen 1988, Evans et al 1989b, Evans et al 1990b). At least in the human osteoblastic cell system, these cytokines may possibly be exerting a de-differentiating effect on osteoblasts associated with concomittant cell proliferation. IL-6, however, had no effect on either basal or $1,25(OH)_2D_3$-induced synthesis of alkaline phosphatase activity or osteocalcin by human trabecular bone cells. Basal levels of alkaline

phosphatase were much higher in ROS 17/2.8 cells compared to human osteoblast-like cells as this was one of the selection markers used when isolating and cloning the ROS cell series. $1,25(OH)_2D_3$ responses, although present, were not as marked in ROS 17/2.8 cells. This could have reflected the density and maturity of the rat osteosarcoma cultures (Majeska and Rodan 1982). Majeska and Rodan (1982) demonstrated that in mature ROS 17/2.8 cultures where alkaline phosphatase activity was high, $1,25(OH)_2D_3$ exhibited a biphasic effect, with a concentration of 10^{-8} M $1,25(OH)_2D_3$ stimulating the enzyme activity in agreement with the results presented in this chapter. However, in the present studies IL-6 did not modulate either the basal or $1,25(OH)_2D_3$-stimulated alkaline phosphatase levels. Also in accordance with the studies presented in this chapter, IL-6 exerted no effect on the alkaline phosphatase activity of UMR 106-01 cells (Fang and Hahn 1991). However, there are other contrasting studies. Ishimi et al (1990) observed that IL-6 slightly suppressed the alkaline phosphatase activity in MC3T3-E1 cells, whereas Hughes and Howells (1992) observed that IL-6 at very high concentrations was able to induce alkaline phosphatase in rat calvarial osteoblasts.

The studies presented in this chapter examining the effects of IL-6 on human osteoblast-like cells and ROS 17/2.8 cells, would suggest that the osteoblast does not appear to represent the primary target cell within the bone microenvironment. Although the effects of IL-6 on collagen synthesis and other matrix proteins have not been studied in this chapter, Linkhart et al (1991) recently demonstrated that IL-6 did not modulate collagen synthesis in normal human osteoblasts. Other studies would suggest that IL-6 may slightly depress collagen synthesis in various osteoblast systems (Ishimi et al 1990, Fang and Hahn 1991). It is possible that IL-6 does exert effects on human osteoblasts, but on a different range of features not measured in this study.

There is evidence to suggest that an osteoclast precursor is responsive to IL-6 however, the effect of IL-6 on bone resorption still remains controversial. Barton and Mayer (1990) demonstrated that IL-6 exerted no effect on bone resorption in neonatal mice calvariae. In these series of experiments, the mothers were allowed to deliver their litter

before sacrificing the 1 day old pups and labelling the calvariae for 48 h with ^{45}Ca. The effects of the interactions of IL-6 with other agents on bone resorption were not investigated in these studies. Al-Humidan et al (1991) also showed that IL-6 alone did not affect resorption in ^{45}Ca-prelabelled neonatal mouse calvariae. Here newborn mice were injected subcutaneously with ^{45}Ca and sacrificed 4 days later before dissection of calvaria. However, this group demonstrated that IL-6 exerted an inhibitory effect on PTH- and $1,25(OH)_2D_3$-stimulated bone resorption but not that induced by IL-1. Addition of an anti-IL-6 antibody blocked the inhibitory effect of IL-6 on resorption stimulated by PTH and $1,25(OH)_2D_3$. This is in contrast to work by Black et al (1990) who demonstrated that IL-6 did not affect bone resorption stimulated by $1,25(OH)_2D_3$ and PTH although it enhanced resorption stimulated by TNF and IL-1. They observed that an anti-IL-6 antibody could inhibit bone resorption stimulated by IL-1 and TNF. The reason for the discrepancy is unknown since the same bone resorption assay model was utilised, but could be a result of using slightly different culture conditions and different preparations of IL-6 and anti-IL-6 antibody. Ishimi et al (1990) also demonstrated that IL-6 increased bone resorption in fetal mouse calvariae from 16 day ^{45}Ca injected pregnant mice killed 1 day later. IL-1 and IL-6 at suboptimal doses synergistically induced bone resorption and osteoclast number on the trabecular bone surface. Several other groups have studied the effects of IL-6 on bone resorption using other experimental systems. Lowik et al (1989) observed that in systems which contain very early osteoclast progenitors such as 17 day old fetal mouse metacarpals, IL-6 stimulates bone resorption. However in 17 day old fetal mouse radii already containing mature osteoclasts, they found that IL-6 did not exert any effect on bone resorption. This suggests that IL-6 may be involved in the recruitment of osteoclast precursors rather than the activation of mature osteoclasts. Evidence for this is further substantiated by the observation that IL-6 increases the formation of multinucleated osteoclast-like cells in long-term human bone marrow culture as defined by reactivity to an antibody 23C6, that recognises osteoclasts (Kurihara et al 1990). Furthermore, IL-6 did not modulate the $1,25(OH)_2D_3$-stimulated multinucleated cell formation but did

increase the percentage of 23C6 positive cells within these cultures. A specific anti-IL-1 antibody also blocked IL-6-induced multinucleated cell formation and the number of 23C6 positive cells. It would therefore appear as though IL-6 is exerting quite complex effects and is almost certainly acting in concert with other factors within the bone microenvironment. The above evidence would indicate that IL-6 may be exerting its effects on an immature osteoclast precursor rather than on mature osteoclasts, although this does not explain all of these observations. The different strains of mice, culture conditions and slight variations in experimental design are quite likely to contribute to these variations.

Relatively little work has been performed to study the function of IL-6 on bone remodelling *in vivo*. However, Black et al (1991a) have demonstrated a hypercalcaemic effect of IL-6 *in vivo*. This was shown by transfecting IL-6 cDNA into Chinese hamster ovarian cells which were then injected into tumour bearing nude mice. After 15 days, the serum ionized calcium levels were outside the normal range and by 19 days serum calcium levels were significantly elevated above control values. In further studies, Black et al (1991b) observed that antibodies to IL-6 reversed the induction of hypercalcaemia in nude mice bearing tumours producing IL-6 and TNF. It is possible that the hypercalcaemia observed in these nude mice was due to an increase in bone resorption although this was not described histologically and so the evidence is therefore circumstantial.

Another well characterised activity of IL-6 is its involvement in hematopoiesis. Therefore hematopoietic stem cells are another potential target for IL-6 within the bone microenvironment. Different systems such as 5-fluorouracil treated mouse spleen cells (Ikebuchi et al 1987, Koike et al 1988, Wong et al 1988, Suda et al 1988), mouse bone marrow cells (Suda et al 1988) and human marrow cells (Hoang et al 1988, Ogawa and Clark 1988, Leary et al 1988) have been used to study the effect of IL-6 on hematopoiesis. Using myeloid leukemia M1 cells, several groups have observed that IL-6 directed the differentiation of M1 cells down the macrophage lineage (Chen et al 1988, Miyaura et al 1988, Miyaura et al 1989a, Metcalf 1989). These macrophages

were shown to possess several macrophage characteristics such as phagocytic activity, adherence to the culture vessel and surface markers (Miyaura et al 1989a). Murray et al (1990) demonstrated presence of LIF and IL-6 mRNA and protein but not GM-CSF or IL-3 in mouse blastocysts at 3.5 days of gestation and surmised that IL-6, together with LIF, may be involved in early hematopoiesis. Suematsu et al (1989) fused the IL-6 gene with an IgG heavy chain enhancer and obtained transgenic mice with elevated serum levels of IL-6 and IgG. In addition, Suematsu and co workers (1989) observed that there was an increase in megakaryocytes in the bone marrow which indicates a hematopoietic role for IL-6. That IL-6 is involved in hematopoiesis is well established and the majority of evidence demonstrates that this cytokine favours the differentiation of lineages, such as monocytes and macrophages, which may ultimately lead to osteoclast generation (Ikebuchi et al 1987, Hoang et al 1988,Wong et al 1988). In support of this theory, IL-6 has been reported to increase the number of osteoclast-like cells in long-term human marrow culture (Kurihara et al 1991). IL-6 appeared to induce the differentiation of a committed (unipotential) precursor and increase the proliferation of early GM-CFU bipotential precursors which can subsequently differentiate into osteoclasts or macrophages (Kurihara et al 1991).

Therefore, IL-6 rather than acting on the bone forming cells, may play a role in bone resorption and hematopoiesis and hence could be important for potential growth and maturation of the osteoclast precursors.

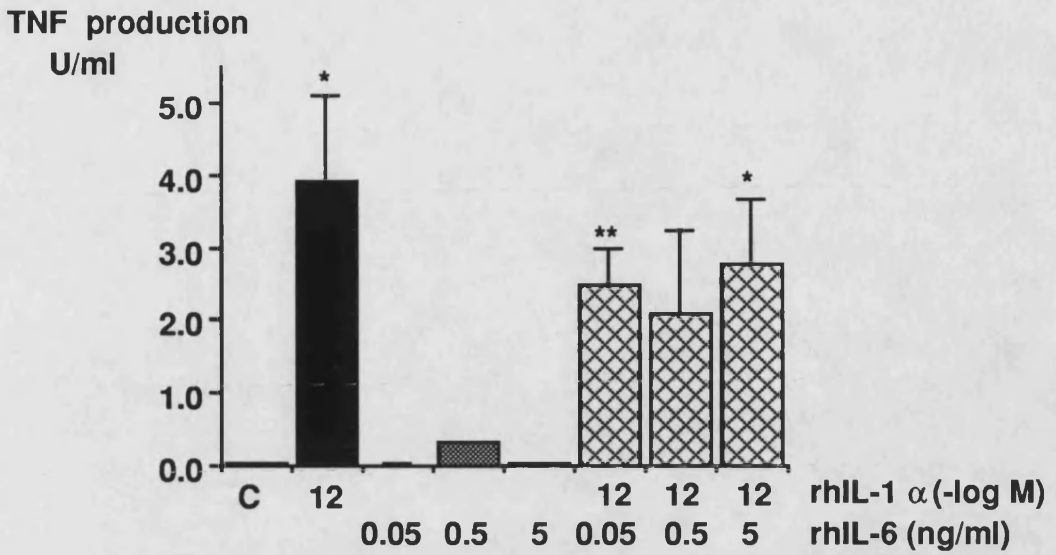


Fig. 3.1 The effect of IL-6 on basal and rhIL-1 -stimulated levels of TNF activity in human osteoblast-like cells.

Human trabecular bone cells were incubated in the presence of agents under test for 6 h in 10% (v/v) FCS-containing EMEM and indomethacin (1.4 uM). The bone cell conditioned medium was removed and assayed for TNF activity (see methods). Mean ± S.E.M. Representative experiment (n=10). Significant difference from unstimulated levels * $p<0.05$, ** $p<0.01$.

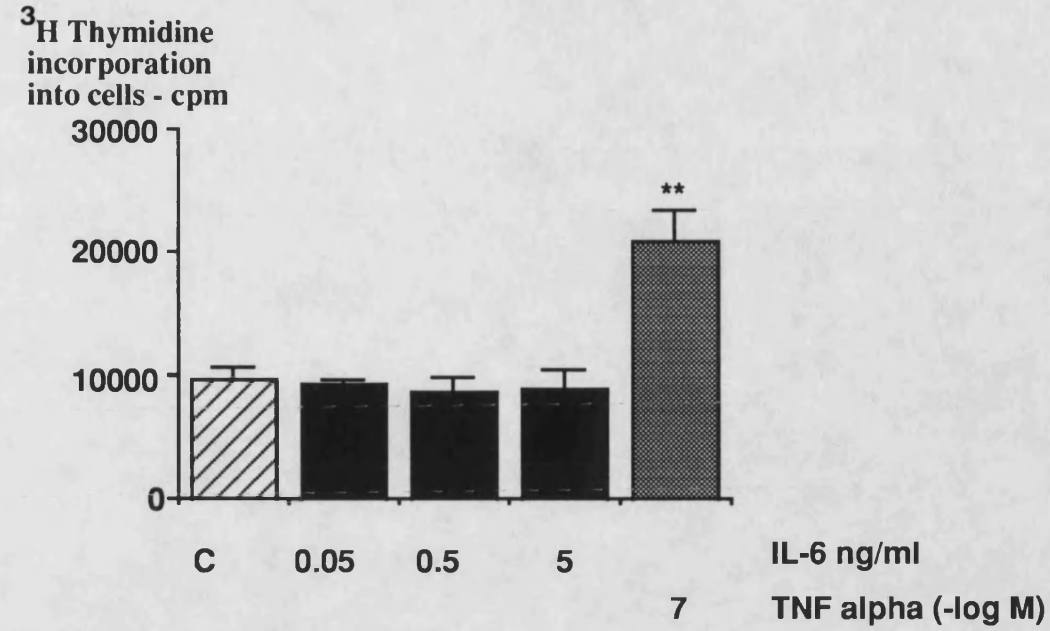


Fig. 3.2 The effect of IL-6 on basal levels of IL-1 activity in human osteoblast-like cells.

Human trabecular bone cells were incubated with the agents under test for 24 h in the presence of 10% (v/v) FCS containing EMEM and 1.4 uM indomethacin. The bone cell conditioned medium was removed and assayed for IL-1 bioactivity (see methods). Mean ± S.E.M. Representative experiment (n=3). Significant difference from unstimulated levels ** $p<0.01$.

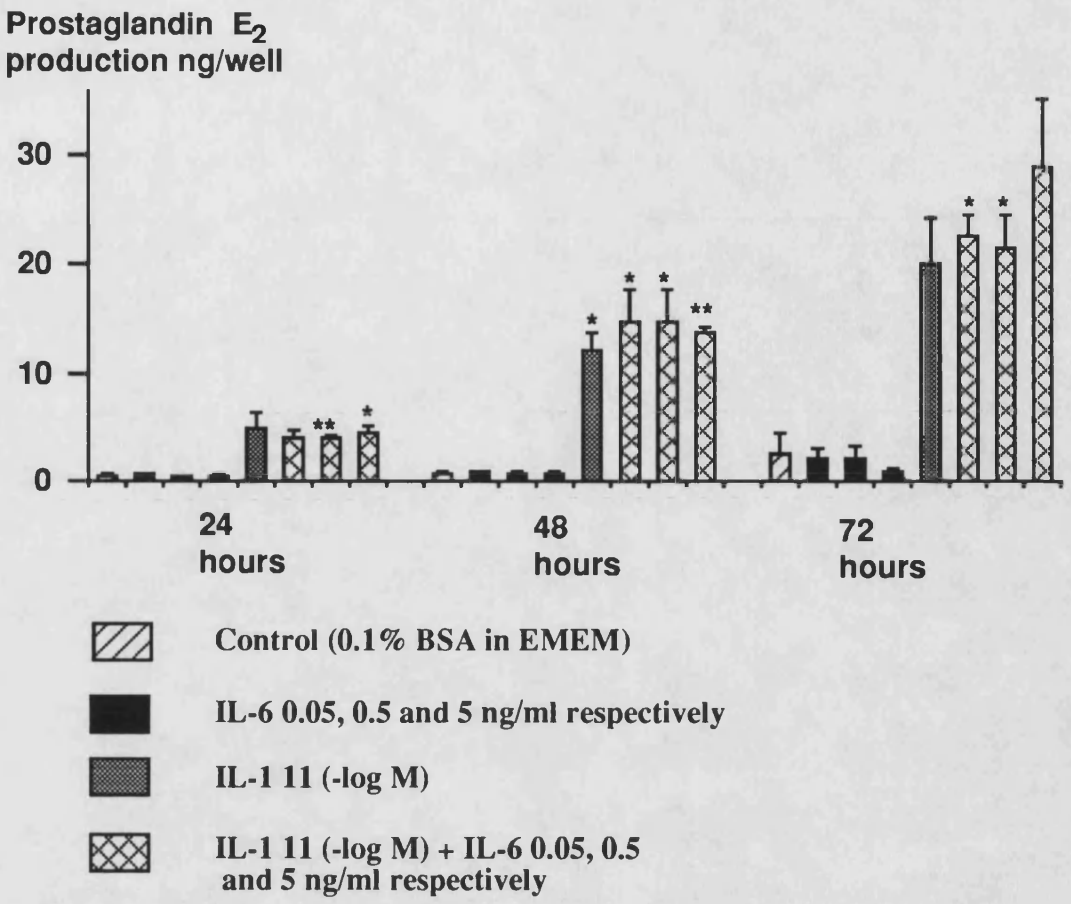


Fig. 3.3 The effect of IL-6 on basal and rhIL-1α-stimulated levels of prostaglandin E activity in human osteoblast-like cells.

Human trabecular bone cells were incubated with the agents under test for 24, 48 and 72 h in the presence of 0.1%(w/v) BSA-containing EMEM. The bone cell conditioned medium was removed and assayed for PGE_2 activity (see methods). Mean ± S.E.M. Representative experiment (n=3). Significant difference from unstimulated levels
* $p<0.05$, ** $p<0.01$.

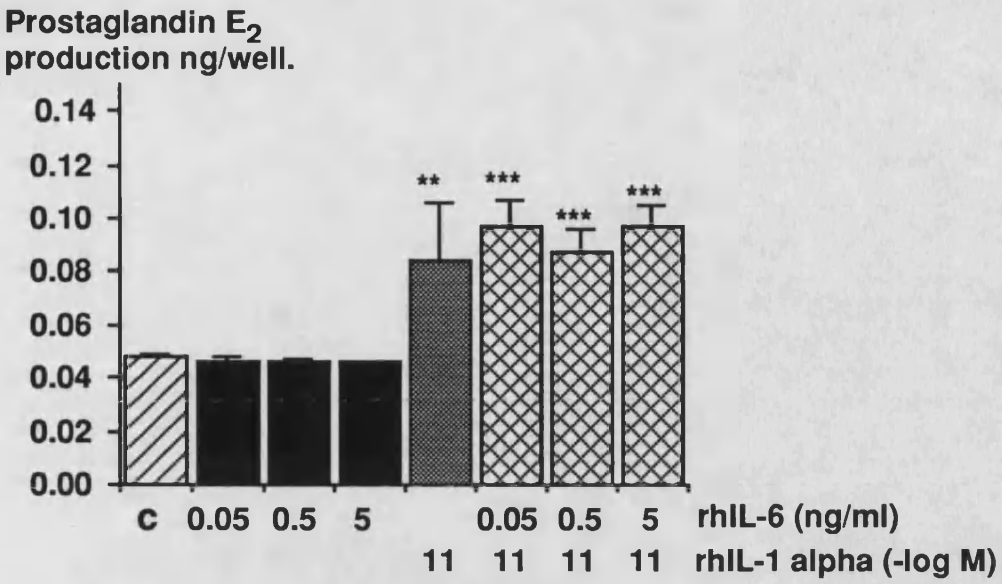


Fig. 3.4 The effect of IL-6 on basal and rhIL-1α-stimulated levels of PGE_2 activity in ROS 17/2.8 cells.

Rat osteosarcoma ROS 17/2.8 cells were cultured with the agents under test for 72 h in the presence of 0.1% (w/v) BSA-containing HAMS F10. The conditioned medium was removed and assayed for PGE_2 activity (see methods). Mean ± S.E.M. Representative experiment (n=3). Significant difference from unstimulated levels **p<0.01, ***p<0.001.

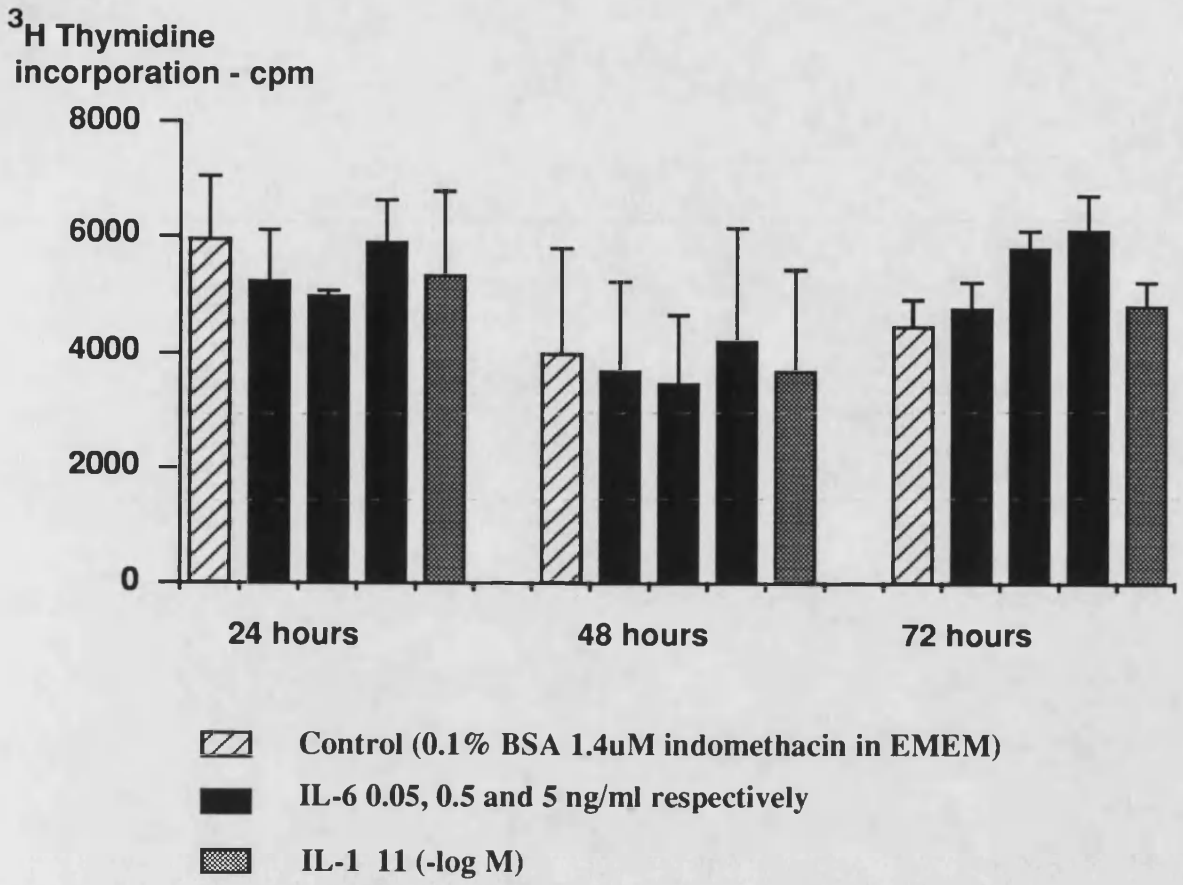


Fig. 3.5 **The effect of IL-6 on basal levels of human osteoblast-like cell proliferation.**

Human trabecular bone cells were cultured in the presence of the agents under test for 24, 48 and 72 h in the presence of 0.1% (w/v) BSA-containing EMEM and 1.4 uM indomethacin. The cell layer was assessed for proliferation (see methods). Mean ± S.E.M. Representative experiment (n=3).

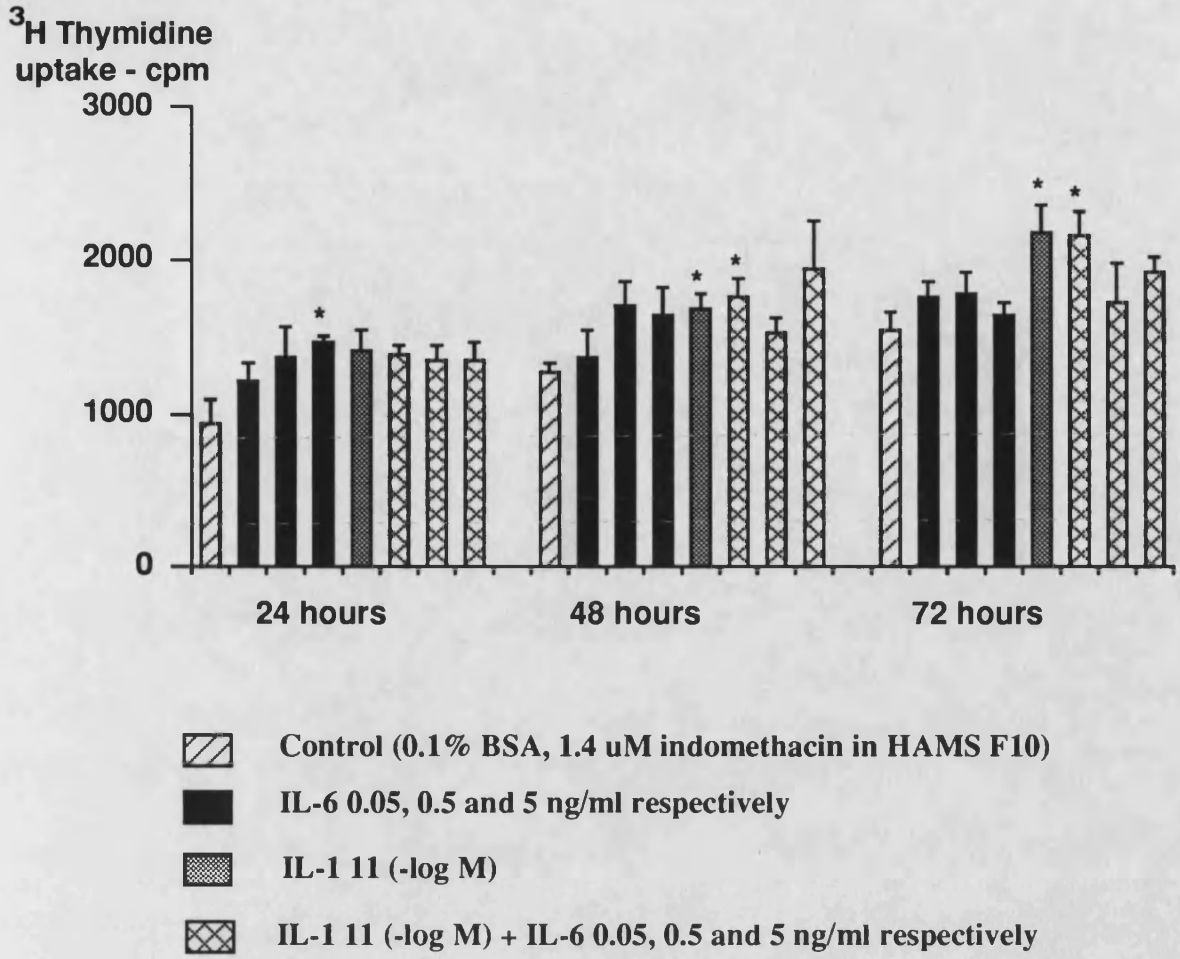


Fig. 3.6 **The effect of IL-6 on basal and rhIL-1α stimulated levels of proliferation in ROS 17/2.8 cells.**

ROS 17/2.8 cells were incubated with the agents under test for 24, 48 and 72 h in the presence of 0.1% (w/v) BSA-containing HAMS F10 and indomethacin (1.4 uM). The cell layer was assessed for proliferation (see methods). Mean ± S.E.M. Representative experiment (n=3). Significant difference from unstimulated levels *p<0.05.

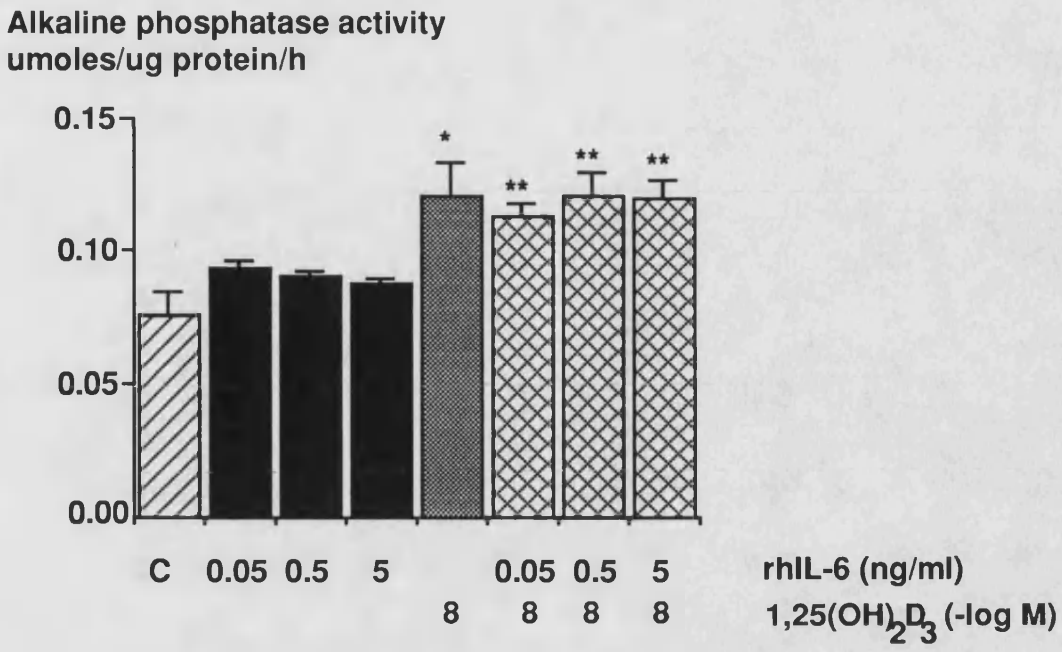


Fig. 3.7 The effect of IL-6 on basal and $1,25(OH)_2D_3$ stimulated levels of alkaline phosphatase activity in human osteoblast-like cells.

Human trabecular bone cells were incubated with the agents under test for 72 h in the presence of 10% FCS-containing EMEM. The cell layer was assessed for alkaline phosphatase activity by measurement of p-nitro phenol release (see methods). Mean ± S.E.M. Representative experiment (n=3). Significant difference from unstimulated levels *$p<0.05$, ** $p<0.01$.

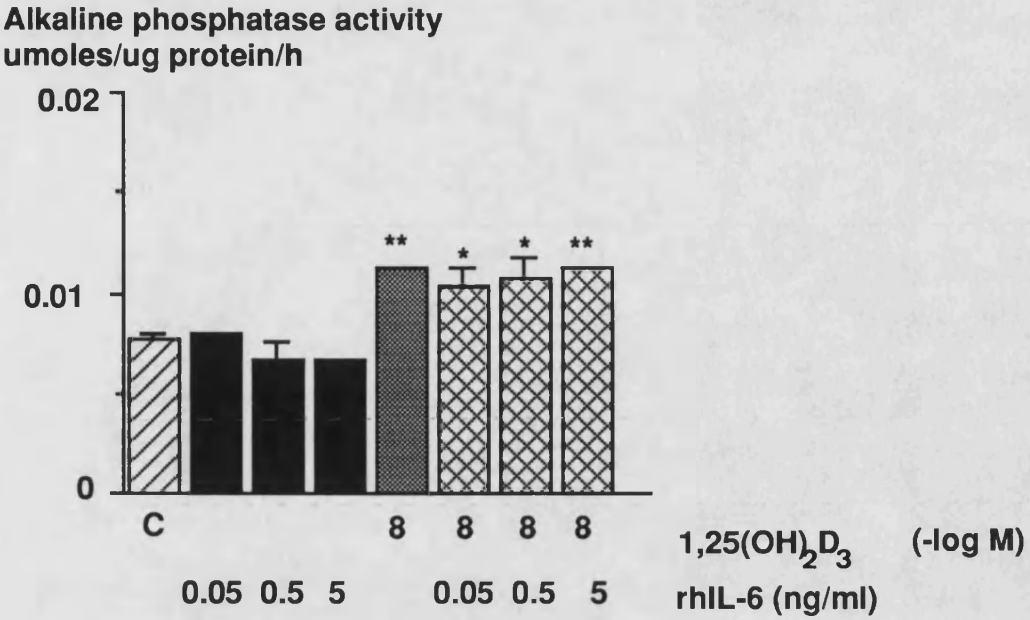


Fig. 3.8 The effect of IL-6 on basal and $1,25(OH)_2D_3$-stimulated levels of alkaline phosphatase activity in human osteoblast-like cells.

Human osteoblast-like cells were incubated with the agents under test for 72 h in the presence of 0.1% (w/v) BSA-containing EMEM. The cell layer was then assessed for alkaline phosphatase enzyme activity by release of p-nitro phenol (see methods). Mean ± S.E.M. Representative experiment (n=3). Significant difference from unstimulated levels *p<0.05, **p<0.01.

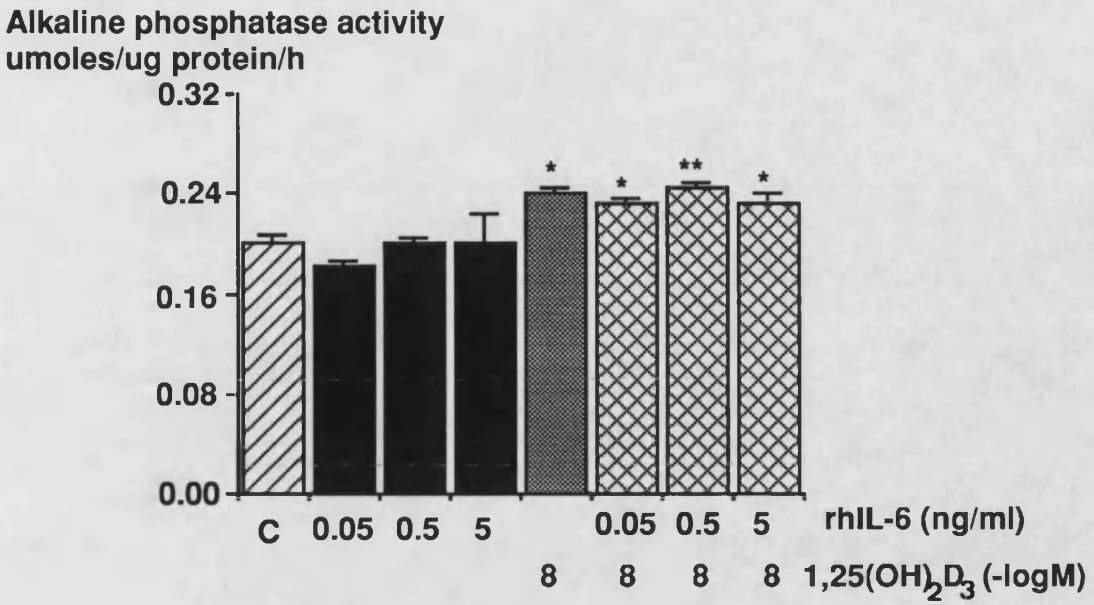


Fig. 3.9 The effect of IL-6 on basal and $1,25(OH)_2D_3$ stimulated levels of alkaline phosphatase activity in ROS 17/2.8 cells.

ROS 17/2.8 cells were cultured with the agents under test for 72 h in the presence of 0.1% (w/v) BSA-containing HAMS F10. The cell layer was assessed for alkaline phosphatase activity by measuring p-nitro phenol release (see methods). Mean ± S.E.M. Representative experiment (n=3). Significant difference from unstimulated levels *p<0.05, ** p<0.01.

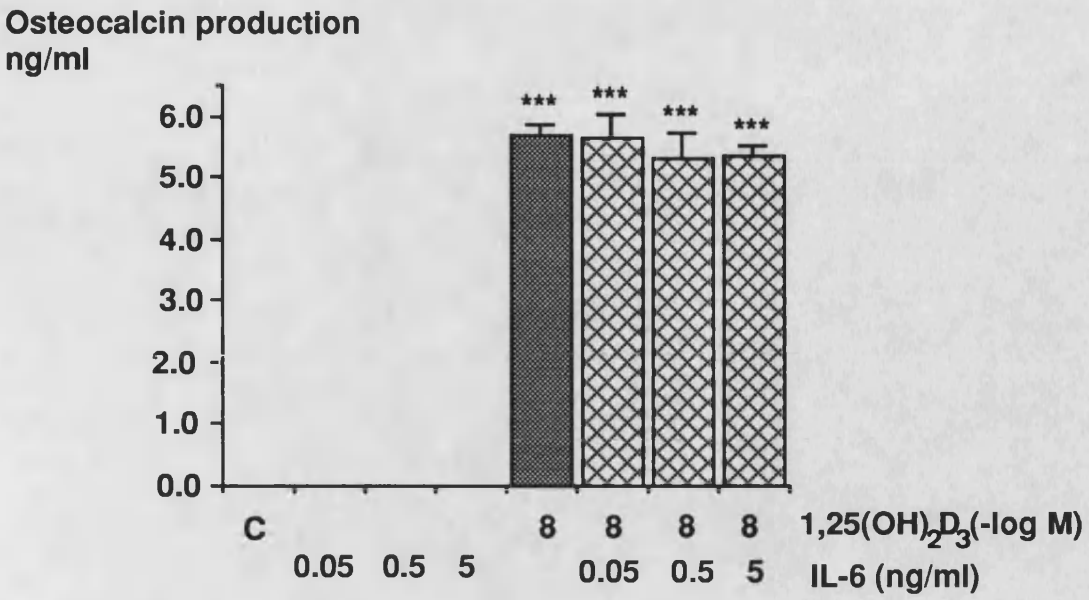


Fig. 3.10 The effect of IL-6 on basal and $1,25(OH)_2D_3$-stimulated levels of osteocalcin production in human osteoblast-like cells.

Human trabecular bone cells were incubated in the presence of the agents under test for 72 h in 0.1% (w/v) BSA- containing EMEM. The osteoblast conditioned medium was assayed for osteocalcin production (see methods). Mean ± S.E.M. Representative experiment (n=3). Significant difference from unstimulated levels ***p<0.001.

Treatment	EMEM + 0.1 % BSA	EMEM + 3% FCS
Control	4245 ±524	7581 ±852
IL-6 0.05 ng/ml	4695 ±589	8227 ±1048
IL-6 0.5 ng/ml	4553 ±712	8927 ±1123
IL-6 5 ng/ml	4177 ±450	8512 ±967
Control + Indomethacin (1.4 uM)	4035 ±573	9033 ±901
IL-6 0.05 ng/ml + Indomethacin (1.4 uM)	3824 ±632	9145 ±955
IL-6 0.5 ng/ml + Indomethacin (1.4 uM)	4233 ±648	8506 ±779
IL-6 5 ng/ml + Indomethacin (1.4 uM)	4343 ±595	8801 ±1116

Table 3.1 The effect of indomethacin (1.4 uM) on proliferation in the presence of IL-6 and under basal conditions in human osteoblast-like cells.

Human trabecular bone cells were incubated with the agents under test for 72 h in 0.1% (w/v) BSA or 3% (v/v) FCS- containing EMEM. The cell layer was then assayed for proliferation (see methods). Mean ± S.E.M. Each column represents 6 pooled experiments using the same 6 bone donors for both conditions.

APPENDIX 2

Alkaline phosphatase standard curve

Stock solution : p-nitrophenol 10 umoles/ml in dd water.

Stock (ul)	0.1% (v/v) Tween diluent	Standard (umoles/ml)
50	200	2
40	182	1.8
40	210	1.6
40	246	1.4
40	293	1.2
50	450	1.0
50	575	0.8
50	783	0.6
25	600	0.4
25	1225	0.2
	1:1 dilution of 0.2	0.1
	1:1 dilution of 0.1	0.05

Alkaline phosphatase assay

Assay buffer

10.52 g diethanolamine and 400 mg magnesium chloride 6-hydrate were dissolved in distilled H_2O to make a total volume of 1 litre and the pH was subsequently adjusted to pH 10-10.5 using 0.1M HCl. This stock buffer was stored at 4°C.

Protein standard curve

Stock solution: Bovine serum albumin (BSA): 500 ug/ml in ddH_2O.

Stock (ul)	0.1% (v/v) Tween diluent (ul)	Standard (ug protein)
200	0	100
175	25	87.5
150	50	75
125	75	62.5
100	100	50
75	125	37.5
50	150	25
25	175	12.5
15	185	7.5
10	190	5

Protein Estimation

Alkaline copper reagent

This was prepared as a fresh solution and contained

1 ml of copper stock solution: (1% (w/v) tri-sodium citrate dihydrate in 0.5% (w/v) copper sulphate in dd H_2O;

20 ml alkaline stock solution (10% (w/v) anhydrous Na_2CO_3 in 2% (w/v) NaOH in dd H_2O)

79 ml of ddH_2O to obtain a final volume of 100 ml.

Osteocalcin assay

Assay buffer

0.01M Tris pH 7.4	6.055g
0.14M NaCl	40.908g
0.025M EDTA	46.525g
0.1% Tween	5g
0.25% BSA	12.5g

5 litres of 0.01M Tris pH7.4 was made up, then remaining chemicals were added.

Preparation of primary osteocalcin antibody

Primary antibody was diluted to approx 1 : 1100 in assay buffer (dilution should be determined after each iodination: A range of dilutions was used and binding of tracer compared at each dilution- 1:1 ratio of tracer : antibody was required).

Addition of reagents to the tubes for osteocalcin assay

	vol (ul)	vol assay buffer (ul)	vol 1' antibody (ul)	vol ^{125}I osteocalcin (ul)
Total counts	-	-	-	50
Non spec. bind.	50	150	-	50
Max. bind.	-	150	50	50
Sample	50	100	50	50
Standard	50	100	50	50

Prostaglandin assay

Preparation of stock PGE_2

1 mg of PGE_2 was dissolved in 1 ml of ethanol and further diluted in working buffer to 1 ug/ml and stored as 100 ul aliquots at -20°C. The standard was subsequently diluted in medium 1:10 to obtain 100 ng/ml.

Assay buffer

1g of gelatin was added to 200 ml of water and heated gently to dissolve. 100 ml stock buffer and 1 ml of chloroform were added to preserve the assay buffer. The pH was adjusted to pH 7.4 with HCl and the final volume was adjusted to 1 litre. Buffers were stored at 4°C.

Stock buffer

Trizma base 12.1 g

NaCl 81.6 g

Reagents were dissolved in 800 ml dd water, the pH adjusted to 7.4 with HCl and the volume adjusted to 1 litre.

Addition of reagents to the tubes for PGE_2 assay

	sample/ standard (ul)	working buffer (ul)	medium (ul)	Tracer- PGE2 (ul)	Antibody (ul)
Sample	100	-	-	100	100
Standard	100	-	-	100	100
Total counts	-	300	100	100	-
Non spec. bin	-	100	100	100	-
Max. bind.	-	-	100	100	100

CHAPTER 4

MODULATION OF THE EXPRESSION OF IL-6 IN OSTEOBLAST-LIKE CELLS.

ABSTRACT

The release of IL-6 from human trabecular bone cells and rat osteosarcoma cell line ROS 17/2.8 treated with a variety of stimuli has been assessed using the murine B9 hybridoma bioassay. In serum-free medium, unstimulated human osteoblast-like cells constitutively produced IL-6 in the range of 200 - 2050 pg/ml over a 24 h period. Recombinant human interleukin 1α (rhIL-1α) (10^{-13} M - 10^{-11} M), recombinant human tumour necrosis factor α (rhTNFα) (10^{-9} M - 10^{-7} M) and lipopolysaccharide (LPS) (5 - 500 ng/ml) all stimulated the release of IL-6 from human bone cells. In these studies maximal levels of 17,000 pg/ml of IL-6 were observed using the highest concentration of IL-1α. In contrast, $1,25(OH)_2D_3$ (10^{-12} M - 10^{-8} M) and PTH (10^{-11} M - 10^{-9} M) did not stimulate IL-6 release. The bioactivity of the IL-6 released by the primary bone cells was completely neutralised using a specific sheep anti-human IL-6 antibody. In parallel studies, ROS 17/2.8 rat osteosarcoma cells released approximately 50 pg/ml of IL-6 under basal conditions which was increased to a maximum of 900 pg/ml by treatment with PTH (10^{-9} M) following a 24 h incubation period. The cytokines rhIL-1α (10^{-13} M - 10^{-11} M), rhTNFα (10^{-9} M - 10^{-7} M) were less effective than PTH, whilst $1,25(OH)_2D_3$ had no effect.

It was of interest to note the differences in levels of production of IL-6 and the cytokine and hormonal responsive profile between the two osteoblast-like cell models used, with the ROS 17/2.8 cells exhibiting a lower basal production compared to the human cells. Modulation of expression of IL-6 mRNA in human osteoblast cells was examined using a human cDNA probe. IL-6 mRNA was constitutively expressed by human osteoblast-like cells and was increased by rhIL-1α (10^{-11} M) and rhTNFα (10^{-7} M) within 2 h of treatment and this effect was sustained over a 24 h period. $1,25(OH)_2D_3$ (10^{-8} M), IL-6 (2000 pg/ml) and PTH (10^{-9} M) exerted no effects at any time point

examined whilst dexamethasone (10^{-6} M) suppressed both the basal and rhIL-1α- and rhTNFα- induced IL-6 mRNA expression.

These results support the hypothesis that IL-6 is produced locally in human bone by osteoblasts under the direction of other cytokines. This could have implications in bone remodelling, hematopoiesis and systemic responses to local injury.

INTRODUCTION

As well as exerting a variety of actions, IL-6, like IL-1 is produced by a large range of cell types, these include endothelial cells (Norioka et al 1988, Howells et al 1991), monocytes (Aarden et al 1987, Tosato and Pike 1988, Bauer et al 1988), fibroblasts (Content et al 1985, Haegeman et al 1986, VanDamme et al 1987b, Gauldie et al 1987, Kohase et al 1987a, Kohase et al 1987b, Walther et al 1988), T cells and blastoid B cells (Hirano et al 1986), thyroid epithelial cells (Zhang et al 1991), synoviocytes (Guerne et al 1989), macrophages (Horri et al 1988, Waage et al 1990, Evans et al 1991), human osteosarcoma cells (VanDamme et al 1987a), myeloid leukemia cells (Miyaura et al 1989a) and thymocytes (Helle et al 1988a). In addition; the IL-6 gene is transcribed in a number of organs including spleen, liver and kidney, and IL-6 expression is closely associated with IL-1 expression (Tovey et al 1988). Many of these groups demonstrated that IL-6 mRNA and/or protein were induced by IL-1 and TNF (Van Damme et al 1987a,1987c, Guerne et al 1989). Although IL-6 is distinct in structure from IL-1, IL-6 exhibits strikingly similar biological activities (discussed in Chapter 3). This together with the fact that IL-1 further induces IL-6 from many cell types may indicate that IL-1 could be exerting some of its effects via a secondary cytokine such as IL-6.

The osteoblast is thought of by many as the central cell in bone remodelling since it not only lays down the matrix but also may control osteoclast activity via the release of soluble mediators (see Chapter 1). Previous work has demonstrated that IL-6 is produced by murine osteoblast-like cells (Lowik et al 1989, Feyen et al 1989, Li et al 1991), synovial cells (Guerne et al 1989) and chondrocytes (Guerne et al 1990,

Bunning et al 1990), an indication that IL-6 may be involved in connective tissue responses.

The aim of the work presented in this chapter was to study the regulation of IL-6 protein and IL-6 mRNA by human and rat osteoblast-like cells in response to various osteotropic agents.

METHODS

IL-6 bioassay (B9)

See Chapter 2.

RNA extraction

Gloves were worn for all procedures. All of the solutions were sterile and RNase free by treatment with 0.1% (v/v) Diethyl pyrocarbonate (DEPC).

Confluent cultures of human osteoblast-like cells, grown in 9 cm petri dishes, were used for mRNA experiments. A separate plate was required for each treatment or control and time point.

The confluent osteoblast cell layer was passaged by trypsinisation, centrifuged (1000 rpm 7minutes) and the cell pellet was suspended in EMEM + 10% (v/v) FCS and plated into fresh 9 cm petri dishes at a density of 1 x 10^6 cells/plate. The cells were allowed to adhere for 24 h prior to addition of the test agents or vehicle in EMEM + 10% (v/v) FCS. If the agents were to be tested under serum-free conditions however, the cells were serum depleted for a further 24 h before addition of the test agents.

The test agents or vehicle alone were added for each time point (generally 2, 6 and 24 h).

At each time point, an aliquot of conditioned medium was removed from the cultures and stored at -20°C for IL-6 bioassay. The remaining medium was discarded and the cell layer was dissolved in 0.5 ml solution D (see Appendix 3). The cell layer was scraped thoroughly using a sterile cell-lifter and the contents were transferred to a microfuge tube.

RNA was isolated by phenol/chloroform extraction and further purified by two sequential chloroform washes (see Appendix 3). Following storage of the isopropanol precipitated RNA for at least 3 hours at -20°C, the RNA pellet was redissolved in 150 ul solution D and re-precipitated with an equal volume of isopropanol and stored at -20°C overnight.

The RNA pellet was washed twice with 70% (v/v) ethanol and once with absolute ethanol, dried down under vacuum and redissolved in 50 ul DEPC treated water.

Quantification of RNA

An aliquot of RNA (usually 5 or 10 ul) was diluted in water to a total volume of 250 ul.

The absorbance was read in a silica cuvette at 260 nm and 280 nm and the ratio of absorbance at 260 nm : 280 nm was determined. A pure RNA preparation should have a ratio of 2 at 260 nm : 280 nm. Contamination by phenol or proteins would lower this ratio.

Concentration of RNA was calculated by:-

$$A_{260} \times 40 \times \text{dilution} = \text{ug/ml RNA}$$

(40 ug/ml of pure RNA has an OD of 1)

Northern blot analysis of RNA

Northern hybridisation is used to assess the size and amount of a specific mRNA species. RNA is separated by electrophoresis according to its size on a denaturing agarose gel and is subsequently transferred onto a nylon membrane. The particular mRNA species is then localised by hybridisation with a specific radiolabelled cDNA probe followed by autoradiography.

After RNA quantification, the equivalent of 10 ug of RNA was reprecipitated in 3 M sodium acetate (0.1 x sample volume) and absolute alcohol (2 x sample volume) overnight at -20°C. Two extra samples were prepared for the purpose of assessing the ribosomal RNA bands on the Northern gel by using ethidium bromide staining.

(Ethidium bromide was not used to stain the whole gel as it hampered the blotting procedure).

The RNA was centrifuged (13,000 rpm for 10 minutes) and the pellet was washed twice in 70% (v/v) ethanol and once in absolute ethanol, dried under vacuum and each sample resuspended in 10 ul of 'loading solution' (see Appendix 3). Samples were denatured at 65°C for 10 minutes, placed on ice and 2 ul RNA loading dye (5 mg bromophenol blue + 0.5 g Ficoll 400 in 2.5 ml DEPC water) added and the samples were loaded immediately into a formaldehyde gel (see Appendix 3).

After sufficient migration of the samples by electrophoresis, the gel was removed and two lanes were carefully cut off for detection of the ribosomal RNA bands (see Appendix 3). The remainder of the gel was set up for Northern blotting (see Appendix 3).

On the following day the blot was carefully disassembled and the transferred RNA on the Hybond N+ filter placed RNA side down on a filter paper soaked in RNase free 50mM NaOH for 5 minutes in order to fix the RNA to the filter. The remaining gel was stained with ethidium bromide in order to ensure that all RNA had been transferred onto the Hybond N+ filter.

The Hybond filter was then washed in 2X SSC (DEPC treated) (see Appendix 3) and wrapped in cling film and stored at 4°C ready for hybridisation.

Dot blotting

This procedure results in a sequential two fold titration of the mRNA thus allowing quantitation between mRNA samples treated with different agents by using a densitometer.

Equal amounts of RNA from each sample were dissolved in DEPC treated water to a final volume of 50 ul. An equal volume of formamide (ultra pure, de-ionised) was then added to each sample tube and the RNA denatured at 65°C for 10 minutes. The sample tubes were placed on ice and 3 M sodium acetate was added to give a final concentration of 0.25 M. Solution A (see Appendix 3) was added to the samples to

adjust the final volume to 200 ul. The samples were then diluted sequentially 1:1 with solution A on ice ready for loading into the prepared dot blot apparatus.

The dot blot manifold was pre-soaked in DEPC treated water for 30 minutes before loading. The Hybond N+ filter membrane, which was used to bind the RNA, was placed on Whatman filter paper soaked in DEPC treated water followed by soaking in the solution 20 X SSC (DEPC treated) (see Appendix 3).

The dot blot apparatus was assembled containing the Hybond N+ membrane and the samples were loaded (lowest dilution first) by drawing the sample RNA slowly through the manifold under pressure generated by a water pump. Each sample well was subsequently washed using 100 ul of solution A.

The apparatus was carefully disassembled and the Hybond N+ filter placed with the RNA side facing down, as for the Northern blot, on paper soaked in DEPC treated 50 mM NaOH for 5 minutes. The Hybond N+ filter was washed in 2 X SSC, wrapped in cling film and stored at 4°C ready for subsequent hybridisation.

Hybridisation of filters

Filters were first pre-hybridised with a pre-treatment solution (see Appendix 3) in order to block non-specific binding of the probe to the Hybond N+ membrane by placing the filter in pretreatment solution in a shaking water bath at 50°C for at least 3 hours.

Labelling of the cDNA probe

Random sequence hexanucleotides are used to prime DNA synthesis on denatured template DNA. These hexanucleotides bind to complementary sites along the full length of the denatured probe. The Klenow fragment (exonuclease fragment removed) of the DNA polymerase I enzyme is used for the incorporation of unlabelled and (α-^{32}P) dCTP in complement to the denatured DNA template.

Denatured cDNA probes (25 ng) were labelled with (α-^{32}P) dCTP using a multiprime labelling kit (Amersham) which utilises the random hexanucleotide method. The cDNA probe was denatured at 100°C for 5 minutes and chilled on ice. Random

hexanucleotides, primer (containing unlabelled nucleotides), radiolabelled (α-^{32}P) dCTP and Klenow enzyme were sequentially added to the denatured cDNA probe.

The labelling procedure was allowed to continue for at least 5 hours at room temperature and the polymerase reaction was stopped using 2 ul of 0.5 M EDTA. Distilled water was added to give a final volume of 200 ul and the incorporation of the ^{32}P into the probe was determined (see Appendix 3). Probes were usually labelled to an excess of 10^8 cpm/ug. If the specific activity was satisfactory, the probe was denatured again at 100°C for 5 minutes and cooled on ice. The labelled probe was then transferred to the prehybridisation mixture and left for hybridisation to the filter overnight at 50°C.

On the following day, the filter was washed once in 2 X SSC (room temperature for 30 minutes) followed by a washing step using 2 X SSC containing 0.5% SDS (two hours at 65°C). The filter was then monitored using a geiger counter and, if required, an optional 0.1 X SSC wash was performed to increase the stringency and subsequent specificity. Following this, the filter was wrapped carefully in cling film and set up for autoradiography. The light-proof autoradiography cassette (+ intensifying screen) containing sensitive film was stored at -70°C until developing (usually 24 h later). The mRNA could be quantitated by recording densitometer readings off the exposed film over a light box and relating the levels to the house-keeping mRNA (β actin).

RESULTS

Release of IL-6 bioactivity by human osteoblasts

Human osteoblast-like cells constitutively released IL-6 when cultured in serum-free and 10% (v/v) FCS-supplemented medium. Concentrations of IL-6 ranged from 200 to 2050 pg/ml and 1950 to 19000 pg/ml in serum-free and 10% (v/v) FCS containing cell conditioned media respectively. After 6 h in serum-free culture, rhIL-1α (10^{-13} - 10^{-11} M) caused a dose dependent release of IL-6. LPS and rhTNFα (10^{-7} M) exerted a slight stimulation of IL-6 synthesis whereas PTH and 1,25$(OH)_2D_3$ had no detectable effects (fig. 4.1). The stimulatory effect of rhIL-1α (10^{-13} M - 10^{-11} M), rhTNFα

(10^{-8} - 10^{-7} M) and LPS (50 - 500 ng/ml) was further enhanced after 24 h in serum-free culture. rhIL-1α was the most potent stimulator at both time points studied with as low as 10^{-13} M rhIL-1α enhancing IL-6 release (fig. 4.2). TNF is generally 100 - 1000 times less potent than IL-1 in its actions on bone *in vitro* (Gowen 1988) and this was reflected by the observation that a concentration of 10^{-8} M rhTNFα was most effective in stimulating IL-6 release in these studies. LPS, a stimulator of cytokine release was used in these studies as a comparison. LPS induced IL-6 production by 24 h, but was less effective than rhIL-1α. The osteotropic hormones PTH and $1,25(OH)_2D_3$ did not affect IL-6 production in human osteoblast-like cells. Parallel studies using human osteoblasts cultured in the presence of 10% fetal calf serum, resulted in the same pattern of expression although levels of IL-6 protein were generally 5 to 50 fold higher in such conditions. After a 6 h incubation, there was little effect of any of the agents on basal IL-6 production (data not shown). However after 24 h culture; rhIL-1α (10^{-12} - 10^{-11} M) stimulated very high levels of IL-6 production. LPS (50 - 500 ng/ml) and rhTNFα (10^{-7} M) were considerably less potent in this respect (fig.4.3).

A quantitative variation in IL-6 production between different patients was observed although similar patterns of stimulation were evident. This appeared to be unrelated to the disease, sex or age of the donor.

A sheep anti IL-6 antibody was used to confirm that the activity in the conditioned medium was due to IL-6 only. (The B9 murine hybridoma assay was sensitive only to IL-6). A 1:1000 dilution of the anti-IL-6 antibody neutralised all IL-6 activity in both the human bone cell conditioned medium and in the standard curve (table 4.1).

Release of IL-6 bioactivity by ROS 17/2.8 cells

Release of IL-6 by ROS 17/2.8 rat osteosarcoma cells was compared with human osteoblast-like cells. The overall production of IL-6 by ROS 17/2.8 cells was much lower than that observed with human bone cells, by at least 10 fold after a 24 h incubation. In 4 experiments the basal range in the amount of IL-6 produced was 17 to

71 pg/ml under serum-free conditions and 247 to 1100 pg/ml using 10% (v/v) FCS-containing HAMS F10.

Under serum-free conditions, after a 6 h incubation, rhIL-1α (10^{-12} M), LPS (5 ng/ml) and PTH (10^{-9} M) showed a slight enhancement of IL-6 release (fig. 4.4). After 24 h serum-free culture, rhIL-1α (10^{-12} M) and rhTNFα (10^{-8} M) exerted only a slight stimulatory effect whereas LPS (5 - 500 ng/ml) enhanced IL-6 release in a dose-dependent manner. $1,25(OH)_2D_3$ had no effect on IL-6 release although PTH had a strong stimulatory effect at (10^{-10} M - 10^{-9} M) in a dose-dependent manner (fig. 4.5). ROS 17/2.8 cells cultured in 10% FCS containing medium showed enhanced production of IL-6 as compared to serum-free culture. PTH (10^{-11} - 10^{-9} M) had a stimulatory effect on IL-6 production in 10% (v/v) FCS-containing HAMS F10 by 6 h and rhTNFα (10^{-9} - 10^{-7} M) and LPS (50 - 500 ng/ml) also induced IL-6 release (fig. 4.6). After a 24 h incubation, PTH (10^{-9} M) was the only agent tested that significantly induced IL-6 release which may infer that factors within the FCS were contributing to the higher basal level of IL-6 expression observed at this time point in ROS 17/2.8 cells (fig. 4.7).

Expression of IL-6 mRNA by human osteoblasts

Using a cDNA probe for IL-6, constitutive expression of an IL-6 mRNA transcript of 1.3 kb was detected in human osteoblast-like cells from all patients tested (n=12). An example from cells obtained from one representative patient is shown in Fig. 4.8.

Under serum-free conditions, rhIL-1α (10^{-11} M) increased IL-6 mRNA levels after 2 h and 6 h with an increase of 17.5 and 38.5 times the constitutive levels (measured by densitometer as described in the methods) at 2 h and 6 h respectively (fig.4.9). This stimulatory effect was sustained over 24 h. The IL-6 protein release was also measured in these cultures and a short lag phase following increased mRNA expression was observed as expected (fig 4.9).

In 10% (v/v) FCS-containing medium, the stimulation of IL-6 mRNA by rhIL-1α (10^{-11} M) was maximal within 4 h (fig. 4.10) which would suggest that other factors within the FCS were contributing to the effects of rhIL-1α.

A marked induction of IL-6 mRNA by rhTNFα (10^{-7} M) was observed under serum-free conditions after 2 h (fig. 4.9 and 4.11). It was interesting to note that rhTNFα exhibited a more marked stimulation of IL-6 mRNA levels after 2 h than rhIL-1α (fig. 4.9). However after 6 h, rhIL-1α appeared to be more potent than rhTNFα. This may indicate that the mechanism of induction of IL-6 mRNA by these two cytokines differs. After 24 h, the enhancement of IL-6 mRNA levels by rhTNFα was still evident. In medium containing 10% (v/v) FCS, the stimulation of IL-6 mRNA by rhTNFα was maximal after 2 h of culture (Fig. 4.12). It would appear from these studies that rhTNFα produced a more rapid accumulation of IL-6 mRNA in comparison to the effects observed with IL-1α (figs 4.12 and 4.10). Northern blot analysis of the induction of IL-6 mRNA by rhTNFα under 10% (v/v) FCS-containing EMEM showed similar effects at 4 h and 8 h although a shorter time point was not included (fig. 4.13). This confirmed that the hybridising species does not alter upon stimulation with various agents.

In contrast to these findings, $1,25(OH)_2D_3$ (10^{-8} M) (fig. 4.14) and IL-6 (2000 pg/ml) (fig. 4.15) did not exert any significant effects on IL-6 mRNA levels under serum-free conditions. The effects of $1,25(OH)_2D_3$ on IL-6 mRNA stimulation in 10% (v/v) FCS-containing EMEM were not studied. PTH (10^{-9} M) also had no modulatory effect under serum-free conditions (fig.4.16) or in the presence of 10% (v/v) FCS (fig. 4.17).

Dexamethasone (10^{-6} M) inhibited basal, rhIL-1α- (10^{-11} M) and rhTNFα- (10^{-7} M) stimulated levels of IL-6 mRNA expression and reduced the IL-6 bioactivity measured in serum-free culture (fig. 4.18 and fig. 4.19).

DISCUSSION

Human osteoblast-like cells synthesized and released IL-6 protein both under serum-free conditions and in the presence of serum. This release could be enhanced by incubation with rhIL-1α, rhTNFα and LPS but not by the hormones PTH or $1,25(OH)_2D_3$. In agreement with the stimulatory effect of IL-1 on IL-6 production in other cell types (Van Damme et al 1987a, 1987c, Helle et al 1988), rhIL-1α was also found to be the most potent stimulator of IL-6 synthesis by the human bone cells. TNF is known to share many of the activities of IL-1 on bone as well as a variety of other tissues (Gowen 1988). It is far less potent than IL-1 in bone cell systems and therefore it is not surprising that the stimulation of IL-6 production in response to TNF was much lower.

ROS 17/2.8 cells have been used as a model system for studying osteoblasts *in vitro* due to their response to PTH with an increase in cAMP and their high levels of alkaline phosphatase and osteocalcin (Majeska et al 1978). IL-6 release in ROS 17/2.8 cells differed from the primary human cells, ROS 17/2.8 cells being more responsive to PTH rather than the cytokines IL-1 and TNF.

There is clearly a qualitative and quantitative difference between human and rodent osteoblasts in terms of the modulation of IL-6 production. From these studies, IL-6 release from human bone cells was not modulated significantly by osteotropic hormones PTH and $1,25(OH)_2D_3$, whilst the cytokines rhIL-1α and rhTNFα appeared to be potent stimulators of IL-6. The differences observed may reflect the possibility that ROS17/2.8 cells were not primary cells, although other groups have studied the production of IL-6 by non transformed mouse calvarial osteoblasts and have also reported responses to PTH (Lowik et al 1989, Feyen et al 1989). It is possible that osteoblast cell maturation may contribute to these observations. Indeed, it has been shown that an 'immature' osteoblast-like cell within rat long bones has the capacity to bind PTH, although more mature, terminally differentiated osteoblasts did not (Rouleau et al 1990). This may indicate that the PTH response could reflect the maturity of the cells in culture. Individual human osteoblast-like cultures, although heterogeneous,

have previously been monitored for an increase in adenylate cyclase activity in response to PTH (MacDonald et al 1986). In the studies presented in this chapter, it was therefore assumed that the lack of response to PTH was not likely to be related to unresponsiveness of the human osteoblast-like cells to PTH but may be related to the number of receptors for PTH or the number of responsive cells in the osteoblast cultures.

The rest of the chapter involved the study of IL-6 mRNA expression in order to investigate whether it parallelled IL-6 protein production and whether the osteotropic agents were acting at the transcriptional level.

In this chapter, IL-6 mRNA levels were unaffected by PTH, IL-6 and $1,25(OH)_2D_3$ whilst rhTNFα and rhIL-1α induced IL-6 mRNA in human trabecular bone cells. Induction was maximal after 6 hours of serum-free culture and was maintained over 24 hours. The studies of Linkhart et al (1991b), who observed that IL-1β, unlike PTH, was capable of stimulating IL-6 mRNA levels in normal human osteoblast-like cells support the data presented in this thesis.

Whether IL-6 mRNA accumulation is a result of increased rate of transcription or stabilization of the IL-6 mRNA species remains to be determined. Elias and Lentz (1990) observed that in human lung fibroblasts, IL-1 and TNF both increased transcription and stabilization of IL-6 mRNA. Further work using actinomycin D, an RNA synthesis inhibitor, is necessary to investigate whether this is the case for the IL-1 and TNF induction of IL-6 mRNA in human osteoblasts.

Other groups, using murine and rat osteoblast systems (Suda et al 1990, Ishimi et al 1990) have also demonstrated that IL-1 and TNF stimulated IL-6 protein and mRNA levels. The observation that the induction of IL-6 mRNA in the presence of 10% (v/v) FCS was faster than in serum-free medium would indicate that FCS contains factors which were acting either in synergy or enhancing the effects of TNF and IL-1. A possible candidate is TGFβ which is present within FCS and induces the production of IL-6 protein from human osteoblast-like cells (K. Merry and A.J. Littlewood unpublished observations). Other workers have demonstrated a similar rate of

induction of IL-6 mRNA with TNF and IL-1 as described here, in fibroblasts and monocytes (Walther et al 1988, May et al 1988). The mechanism of induction of IL-6 mRNA by IL-1 and TNF however appear to be different; IL-1 induction of the IL-6 mRNA was sustained and could be largely blocked by cycloheximide (a protein synthesis inhibitor) whereas the increase in IL-6 mRNA by TNF was more transient and was not blocked by cycloheximide (Walther et al 1988). This group postulated that IL-1 induced a newly synthesized protein that was involved in the transcription of IL-6 mRNA. It was interesting to note in this chapter that TNF consistently induced IL-6 mRNA expression more rapidly than IL-1 in human osteoblast-like cells. These observations may indicate a difference between the induction of IL-6 mRNA by TNF and IL-1. The hypothesis that IL-1 and TNF may regulate IL-6 gene expression via different mechanisms could be elucidated by work on the transcriptional control elements within the IL-6 gene. Comparison of the human and mouse IL-6 genes has revealed highly homologous regions 350 base pairs upstream of the transcription start site (Tanabe et al 1988). Several potential transcriptional control elements such as glucocorticoid responsive elements (GRE), c-fos serum responsive element (SRE), cyclic AMP responsive element (CRE) and AP-1 and NF-kB binding sites have been located within the IL-6 promotor regions of both species (Ray et al 1989). The presence of the SRE may indicate why serum enhanced the induction of IL-6 mRNA at a faster rate than under serum-free conditions in human osteoblast-like cells. Ray et al (1989) also revealed that a 23 base pair multi-responsive element (MRE) within the c-fos SRE may be involved in the induction of IL-6 by TNF, IL-1, serum, forskolin and phorbol ester. Furthermore, Ray and Co-workers (1989) discovered that certain mutations of this 23 base pair MRE, containing an AP-1-like motif, caused a lack of responsiveness to forskolin and phorbol ester. This was in contrast to IL-1, TNF and serum which were still able to react with the MRE. It was therefore postulated by these workers that different sequences within the MRE are important for IL-1, TNF and serum responsiveness. Further studies using other gene mutations/deletion

experiments may show a difference between the effects of IL-1 and TNF induction of IL-6.

Studies involving 5' deletion mutants of the IL-6 gene indicated that an IL-1 responsive element existed which mapped within the IL-6 promoter region containing the c-fos SRE (Isshiki et al 1990), confirming the work of Ray et al (1989). A nuclear factor (NF-IL-6) was identified that recognised a unique palindromic 14 base pair sequence which corresponded to the region homologous to the c-fos SRE (Isshiki et al 1990) which could also be stimulated by IL-1. It would be interesting to study whether TNF and other inflammatory cytokines reacted with this same 14 base pair region. Ray et al (1989) and Isshiki et al (1990) independently observed that NF-kB was not necessary for the IL-1 responsiveness in the IL-6 promoter. This contrasted with the work of another group who demonstrated that NF-kB may also be responsible for IL-6 induction by IL-1 and TNF (Shimizu et al 1990). Shimizu and Co-workers (1990) also showed that TNF induction of the IL-6 gene was more transient than IL-1 induction although they recognized the same 10 base pair sequence (homologous to NF-kB) in accordance with data obtained by Walther et al (1988).

From the above lines of evidence, it is therefore possible that IL-1 and TNF may bind preferentially to different combinations of the various responsive elements within the IL-6 promotor. Alternatively, TNF and IL-1 may recognise common IL-6 promotor elements despite subsequent differences in mechanism of action of these cytokines as a consequence of the induction of different nuclear factors.

In the studies presented in this chapter, dexamethasone inhibited the production of IL-6 by human osteoblast-like cells both at the transcriptional level and at the protein level. Glucocorticoids have been reported to inhibit the production of IL-6 by many other cell types including rat anterior pituitary cells (Carmeliet et al 1991), human monocytes, endothelial cells and fibroblasts (Waage et al 1990). It is likely that dexamethasone is acting on the glucocorticoid responsive element located in the 5' upstream sequence of the IL-6 gene. Almawi et al (1991), demonstrated that dexamethasone inhibited T cell proliferation by blocking the expression of various cytokines including IL-6. Alamwi

and co-workers postulated that dexamethasone interaction with a glucocorticoid responsive region within a cytokine gene may either mask part of the sequence necessary for transcription or initiate transcription of novel regulatory proteins which could subsequently block gene transcription. The inhibitory effects of dexamethasone observed in the study presented in this chapter may indicate a functional role of naturally circulating glucocorticoids in suppressing IL-6 release from IL-6 producing cells. IL-6 is produced by rat anterior pituitary cells and is induced by IL-1 (Spangelo et al 1991) and inhibited by dexamethasone (Carmeliet et al 1991). In addition, IL-6 is reported to increase the secretion of adrenocorticotrophic hormone (ACTH) within 30 minutes in rats (Naitoh et al 1988) and ACTH in turn can increase glucocorticoid levels which subsequently decrease cytokine levels. Therefore a potential complex negative feedback loop may exist in this system. Other groups have observed significant correlations between cortisol levels and the inflammatory activity in rheumatoid arthritis (Neeck et al 1990). This stresses that raised cytokine levels during inflammatory conditions may be capable of inducing the production of immunosuppressive glucocorticoids. One mechanism of action of therapeutically administered glucocorticoids could be to reduce local and circulating levels of inflammatory cytokines such as IL-6.

From the studies presented within this chapter, it is evident that the production of IL-6 in osteoblasts can be modulated by certain bone active agents *in vitro*, although the physiological consequences of this observation still remain to be clarified.

Table 4.1

Neutralisation of the standard curve and human bone cell conditioned medium with a specific sheep anti human IL-6 antibody.

	Δ E 570–630	
Standard Curve pg/ml	absence of antibody	presence of antibody 1:1000 dilution
100	0.349	0.140
30	0.321	0.120
10	0.286	0.111
1	0.222	0.096
0.1	0.159	0.092
0	0.118	0.097
Conditioned medium	0.326	0.156
	0.333	0.125
	0.313	0.114

Known quantities of IL-6 were preincubated with antibody or non immune sheep serum for an hour before addition to the B9 cells (See Chapter 7 Methods). Triplicate samples of conditioned medium from unstimulated human osteoblast-like cells were diluted 1:1000 and preincubated as above. This experiment is representative of eight carried out on different conditioned media.

Fig. 4.1

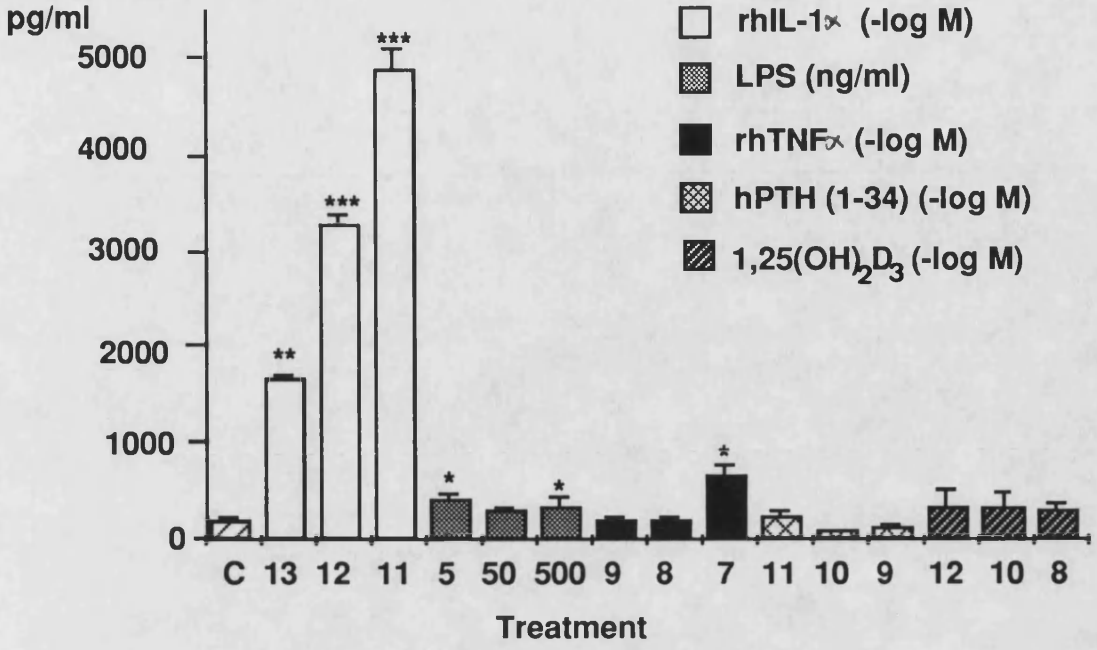
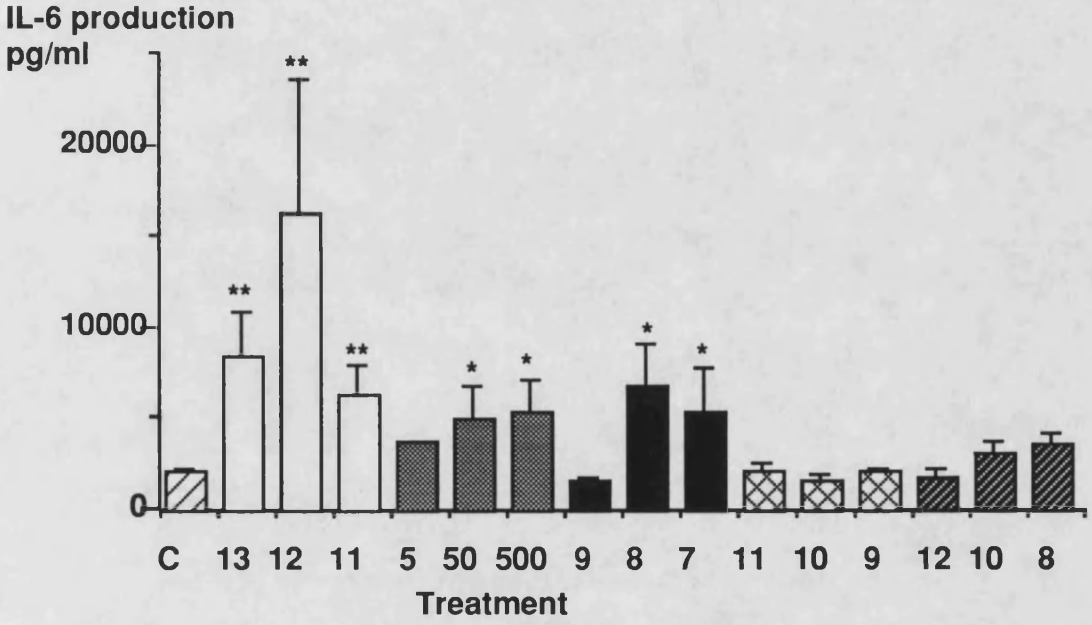


Fig. 4.2



Figs. 4.1 and 4.2. IL-6 production in human osteoblast-like cells after 6 h (4.1) and 24 h (4.2) in serum-free culture.

The trabecular bone cells were incubated with the osteotropic agents shown for 6 h (Fig. 4.1) and 24 h (Fig. 4.2) and the bone cell conditioned medium was assayed for IL-6 activity using the B9 cells (see general methods section). C= 0.1% (w/v) BSA and 1.4 uM indomethacin in EMEM. Representative experiment (n = 4). Significant difference from control *p < 0.05, **p < 0.01, ***p < 0.001.

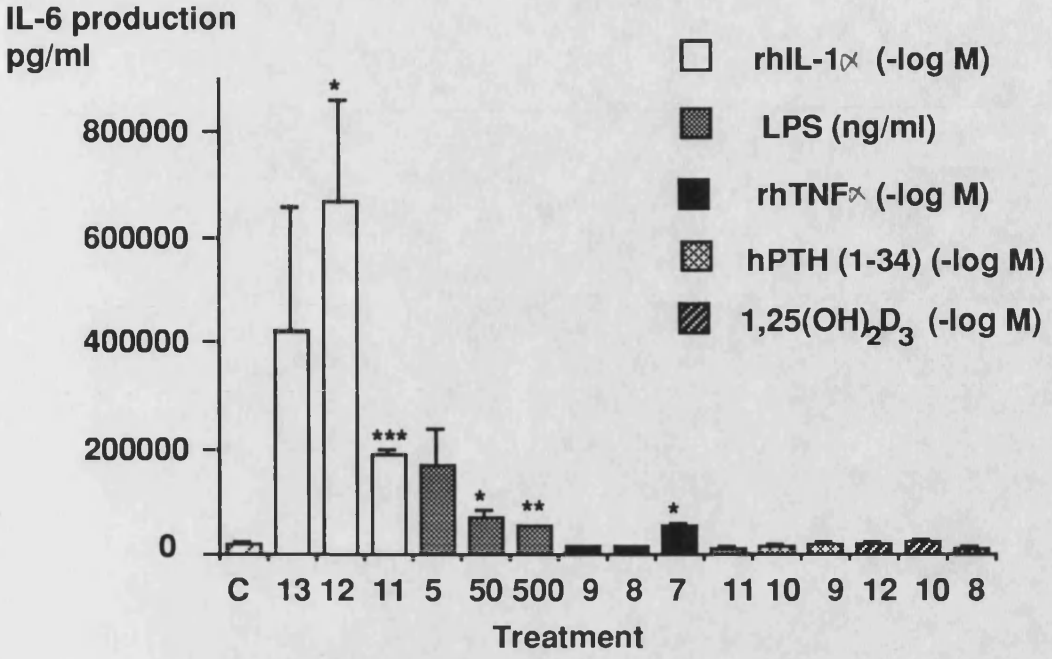


Fig. 4.3. IL-6 production in human osteoblast-like cells after 24 h in 10% (v/v) FCS-containing EMEM.

The human trabecular bone cells were incubated with the osteotropic agents shown for 24 h and the bone cell conditioned medium was removed and assayed for IL-6 activity using the B9 cells (see general methods section). C = 10% FCS and 1.4 uM indomethacin. Representative experiment (n = 4). Significant difference from control *p < 0.05, **p < 0.01, ***p < 0.001.

Fig. 4.4

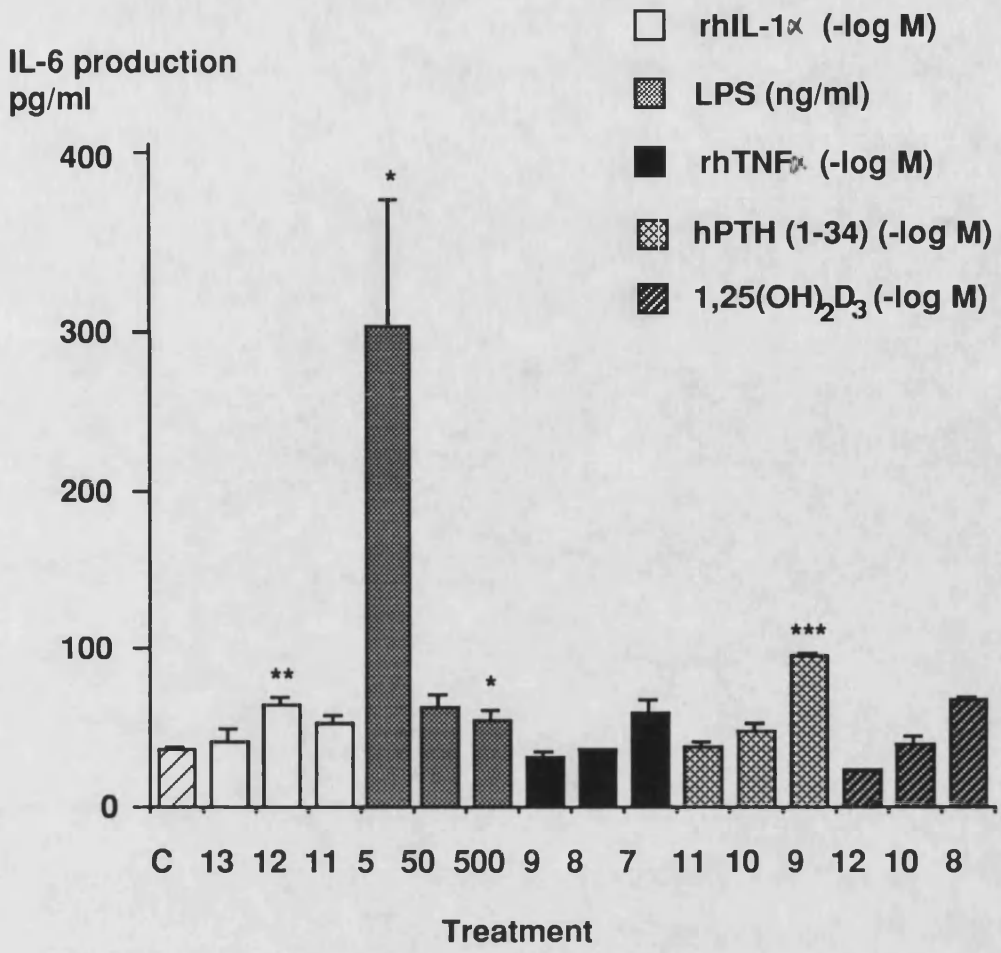
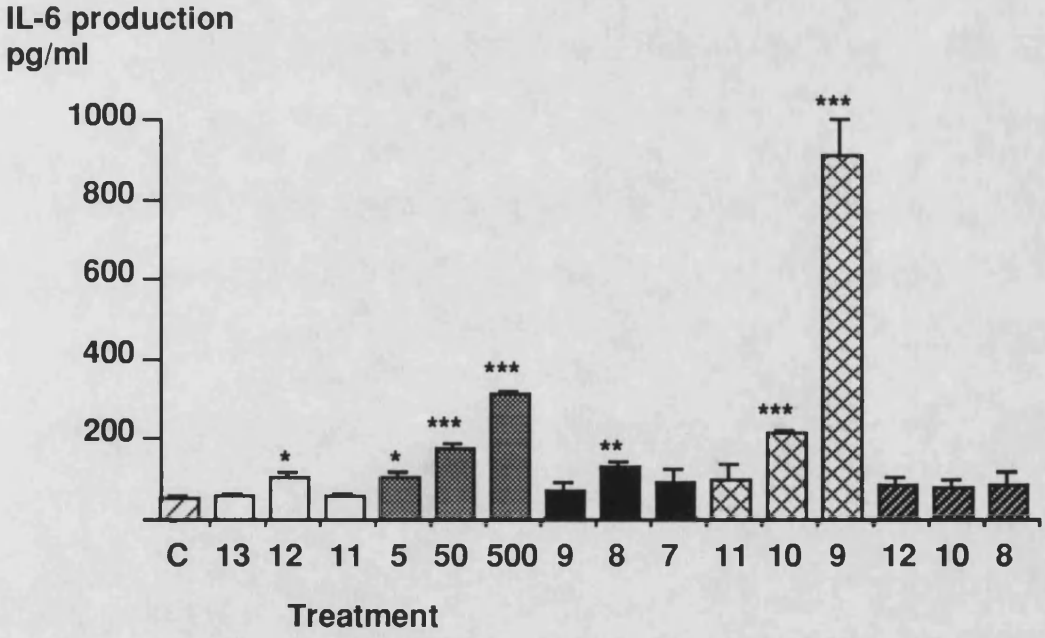


Fig. 4.5



Figs. 4.4 and 4.5 <u>IL-6 production in ROS 17/2.8 cells after 6 h (Fig. 4.4) and 24 h (Fig. 4.5) in serum-free culture.</u>

The rat osteosarcoma cells were incubated with the osteotropic agents shown and the cell conditioned medium was removed at 6 h (Fig. 4.4) and 24 h (Fig. 4.5) for IL-6 bioassay using the B9 cells (see general methods section). C = 0.1% (w/v) BSA and 1.4 uM indomethacin in HAMS F10. Representative experiment (n = 4). Significant difference from control *p < 0.05, **p < 0.01, ***p < 0.001.

Fig. 4.6

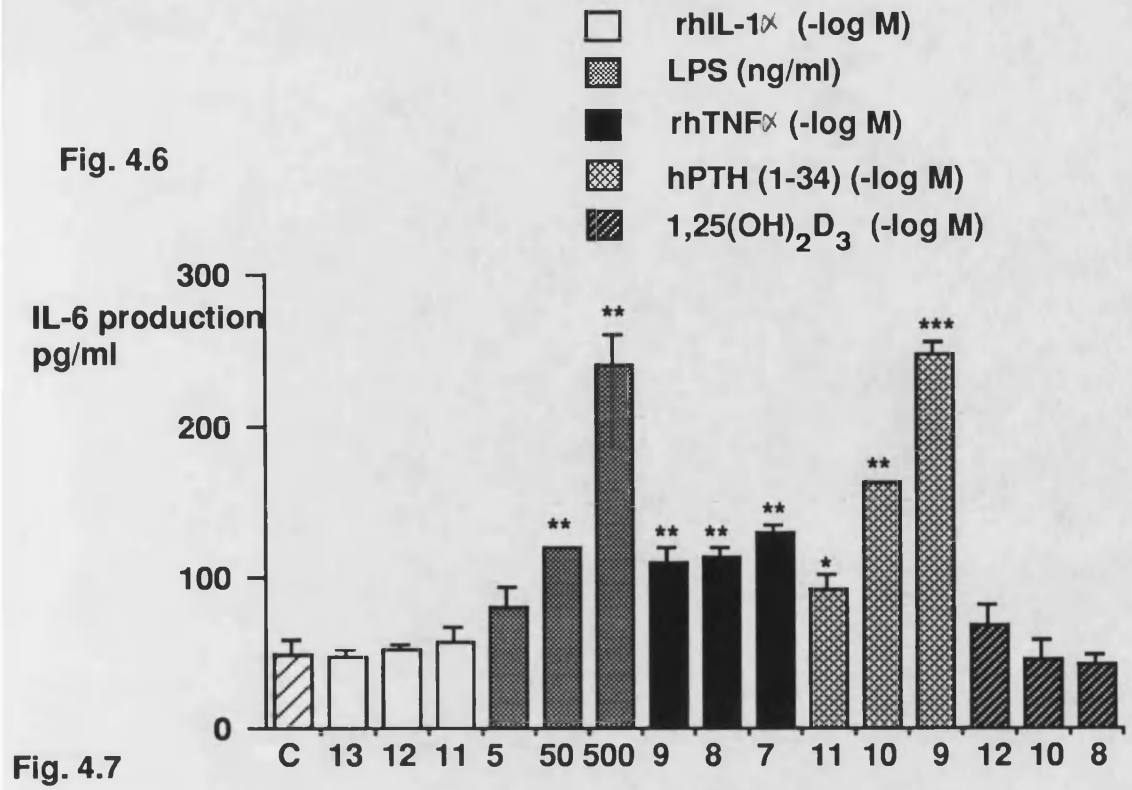
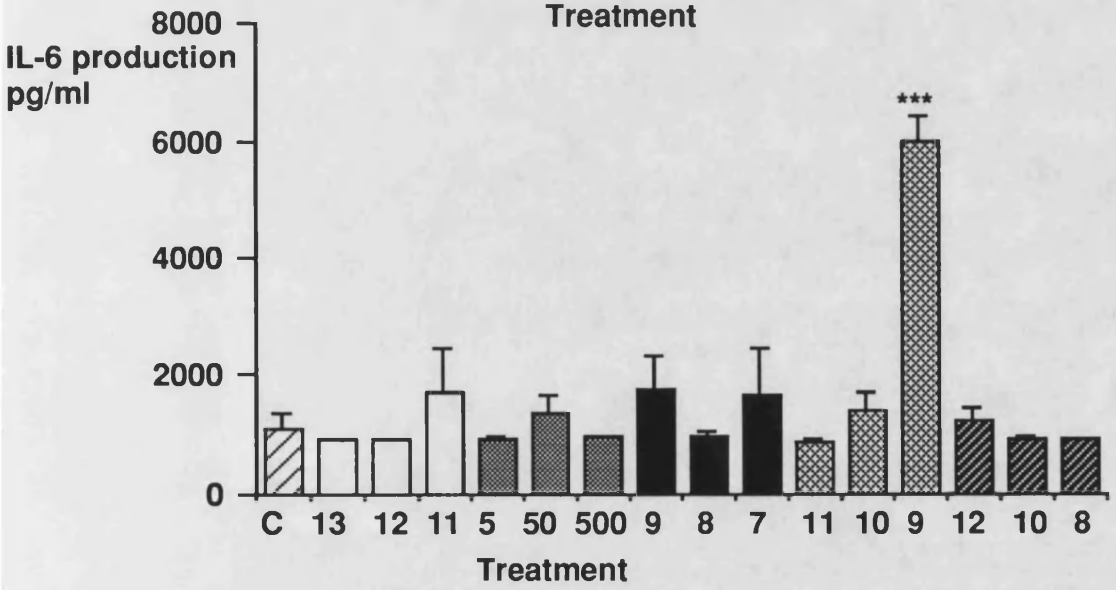


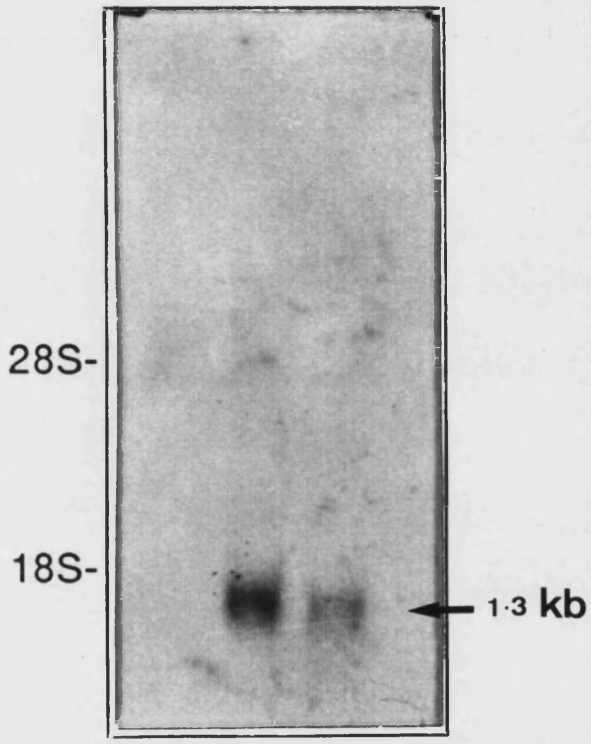
Fig. 4.7



Figs. 4.6 and 4.7 **IL-6 production in ROS 17/2.8 cells after 6 h (Fig. 4.6) and 24 h (Fig. 4.7) in 10% (v/v) FCS-containig EMEM.**

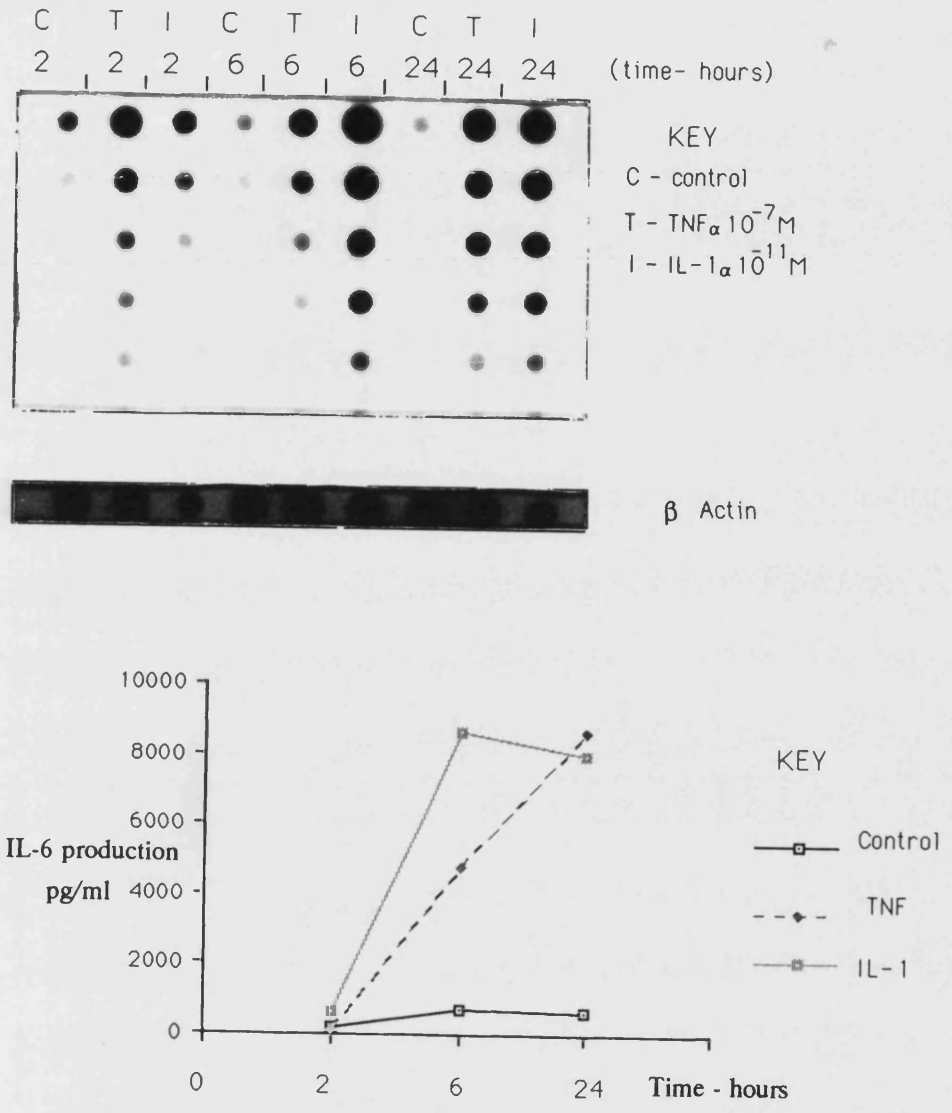
The rat osteosarcoma cells were incubated with the osteotropic agents shown and the cell conditioned medium was removed at 6 h (Fig. 4.6) and 24 h (Fig. 4.7) for IL-6 bioassay using the B9 cells (see general methods section). C = 0.1% (w/v) BSA and 1.4 uM indomethacin in HAMS F10. Representative experiment (n = 4). Significant difference from control *p < 0.05, **p < 0.01, ***p < 0.001.

Fig.4.8. **Constitutive expression of IL-6 mRNA in human osteoblast-like cells by Northern blot.**



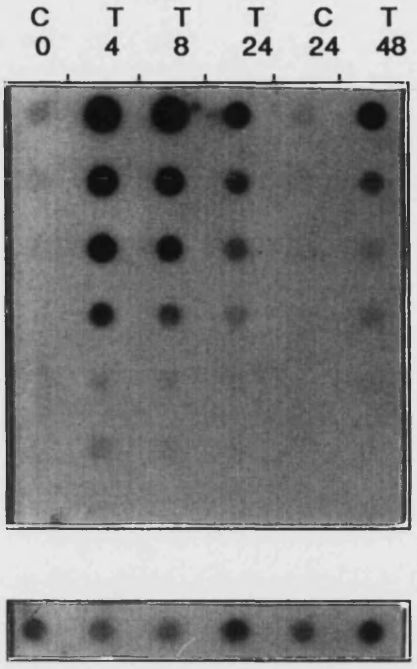
10 ug of RNA from trabecular bone cells was loaded for each of the 2 lanes. The resultant filter was probed for IL-6 mRNA. Ribosomal RNA bands were used as size markers and the IL-6 mRNA transcript was 1.3 kb. This is a representative example of the twelve patients studied.

Fig.4.9. rhTNFα and rhIL-1α induced stimulation of IL-6 mRNA and protein in human osteoblast-like cells under serum-free conditions.



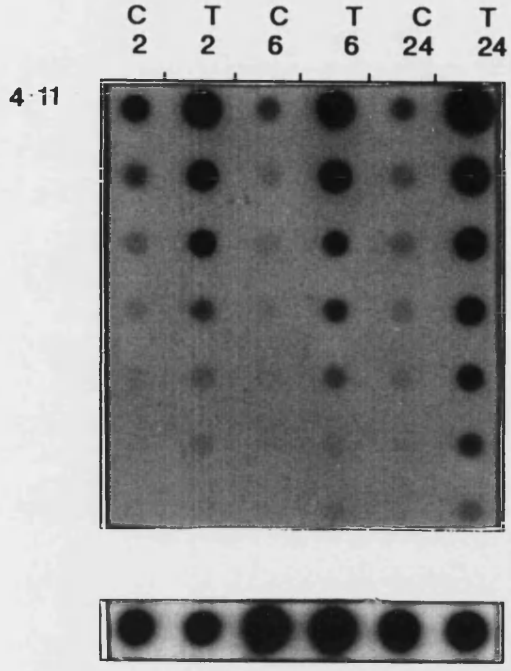
RNA from unstimulated human osteoblast-like cells, and those treated with rhTNFα or rhIL-1α for 2, 6, and 24 h were loaded using doubling serial dilutions starting with a highest concentration of 3 ug. Blots were probed with IL-6 cDNA, stripped and reprobed with β actin to ensure equal loading. IL-6 protein content in the conditioned medium, taken at the same time as the cells were used for RNA extraction, was assayed using the B9 bioassay.

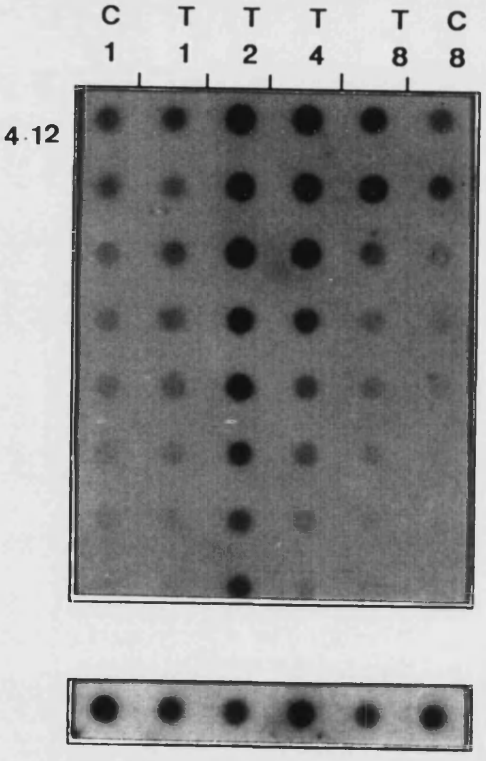
Fig 4.10 Modulation of IL-6 mRNA by rhIL-1α in trabecular bone cells in the presence of EMEM +10% (v/v) FCS.



Human osteoblast-like cells were stimulated with rhIL-1 at a concentration of 10^{-11}M for 4, 8, 24 and 48 h. Total RNA was extracted and doubling dilutions starting at 5 ug of RNA were used and the filter probed for IL-6 and β actin.

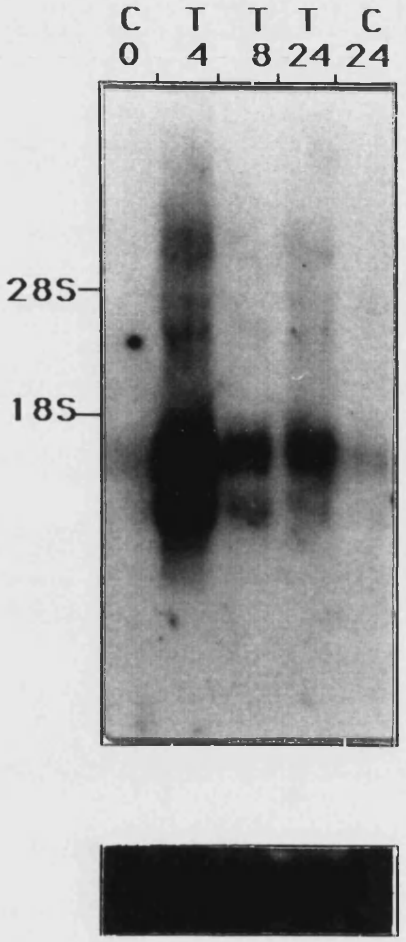
Modulation of IL-6 mRNA levels by rhTNFα in trabecular bone cells in serum-free (fig. 4.11) and 10% (v/v) FCS (fig. 4.12) containing EMEM.





Human osteoblast-like cells were treated with rhTNFα (10^{-7}M) and total RNA was extracted at various time points. Sequential two-fold dilutions were made starting with 4 ug of RNA as the highest concentration. After probing with IL-6, the filter was stripped and reprobed with ßactin.

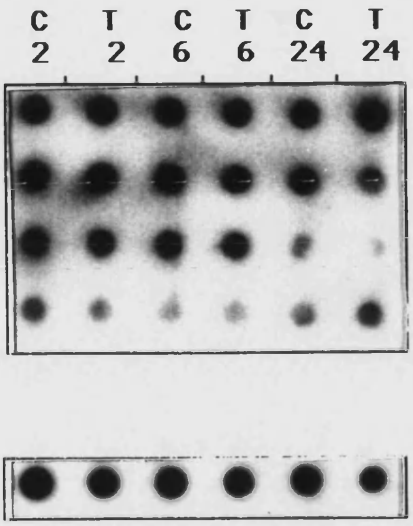
Fig 4.13 Stimulation of IL-6 mRNA in human trabecular bone cells by rhTNF∝ in EMEM containing 10% (v/v) FCS by Northern blot analysis.



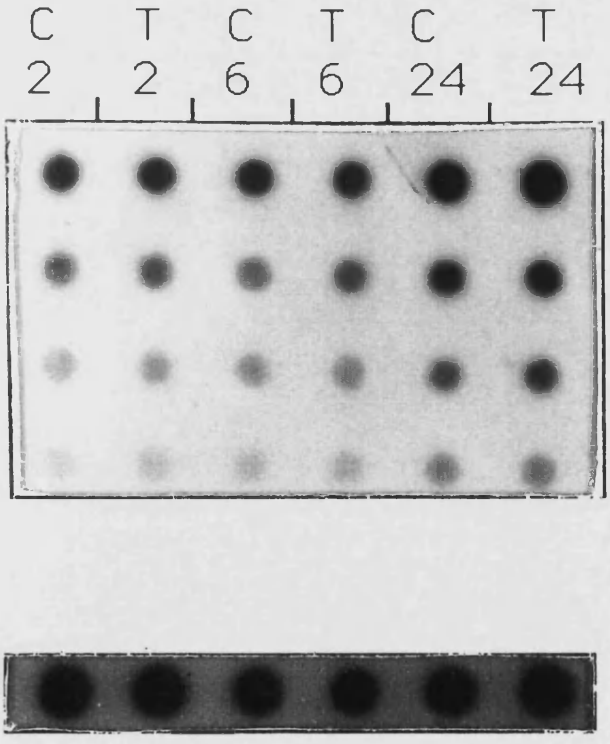
Human osteoblast-like cells were treated with rhTNF∝ (10^{-7}M) for 4, 8 and 24 h and total RNA was extracted. 9 ug of RNA was loaded in each lane. After probing with IL-6, the filter was stripped and reprobed with ß actin. C = unstimulated cells and T = rhTNF∝.

Lack of modulation of IL-6 mRNA in human osteoblast-like cells by $1,25(OH)_2D_3$ (fig. 4.14) and IL-6 (fig. 4.15) in serum-free containing EMEM.

4.14

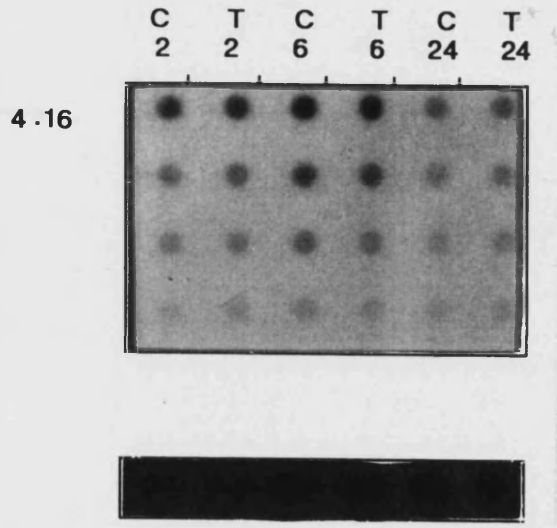


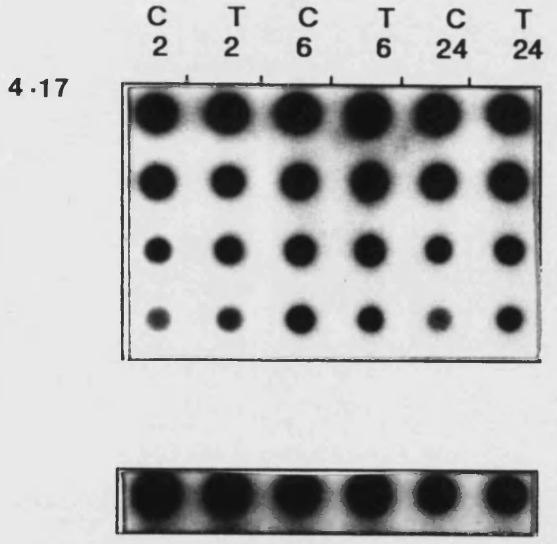
4.15



Human osteoblast-like cells were treated with $1,25(OH)_2D_3$ (10^{-8}M) or IL-6 (2000 pg/ml) for 2, 6 and 24 h and total RNA was extracted. Doubling dilutions starting at 6 ug of RNA were used and the filter probed for IL-6 and βactin. C = unstimulated cells (vehicle) and T = treatment.

Lack of modulation of IL-6 mRNA by PTH in human trabecular bone cells in serum-free (fig. 4.16) and 10% (v/v) FCS (fig. 4.17) containing EMEM.

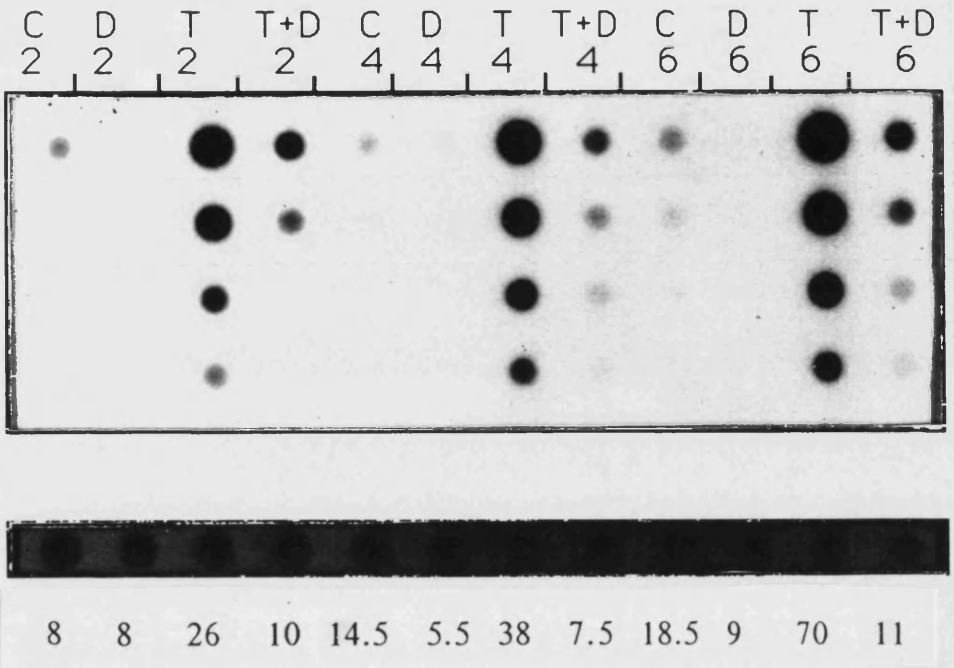




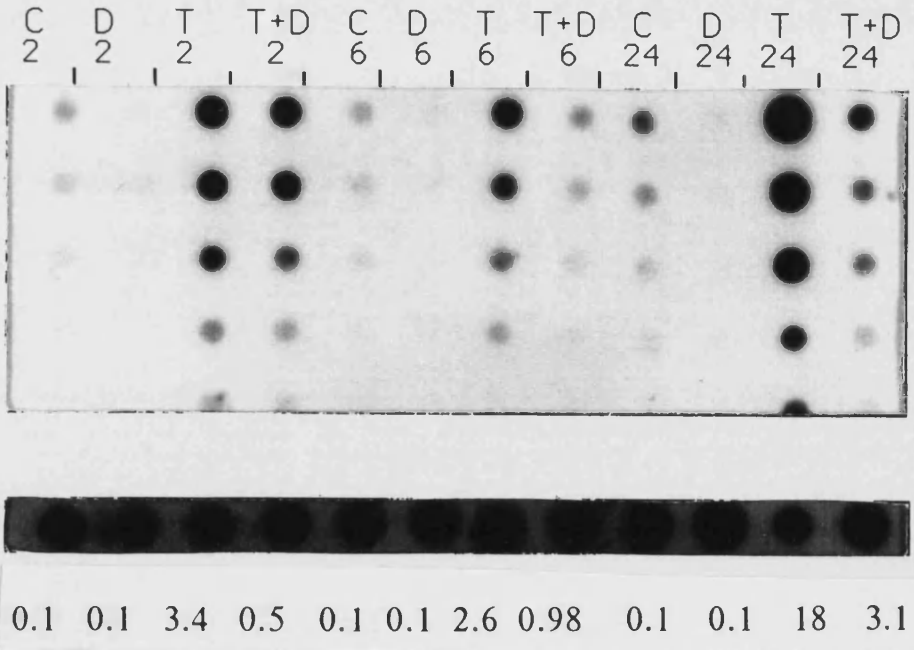
Human osteoblast-like cells were treated with PTH (10^{-9}M) for 2, 6 and 24 h and total RNA was extracted. Doubling dilutions starting with 3 ug of RNA were used. After probing with IL-6, the filter was stripped and reprobed with ß actin. C = unstimulated cells (vehicle) and T = treatment.

Effect of rhIL-1∝(fig. 4.18) and rhTNF∝(fig. 4.19) alone and in combination with dexamethasone on IL-6 mRNA and protein levels in human trabecular bone cells under serum-free conditions.

4.18



4.19



The osteoblasts were treated with rhIL-1∝(10^{-11}M), rhTNF∝(10^{-7}M) alone and in combination with dexamethasone (10^{-6}M) for various time points and the total RNA was extracted. Doubling dilutions starting with 4ug of RNA were used.
The filter was probed for IL-6 mRNA followed by ßactin. At the time of RNA extraction, the conditioned medium was retained for IL-6 bioassay using B9 cells (figures under the ß actin strip). C = unstimulated cells (vehicles), D = dexamethasone and T = treatment (rhIL-1∝ and rhTNF∝).

APPENDIX 3

Denaturing solution - Soln. D

4M guanidinium thiocyanate (disrupts cells and inhibits RNase activity)

25mM sodium citrate, pH 7

0.5% sarcosyl

0.1M 2-mercaptoethanol (2-ME) (inhibits RNases)

To minimize handling of guanidinium thiocyanate, a stock solution was prepared as follows:-

Guanidinium thiocyanate	250g
distilled deionized water	293ml
0.75M sodium citrate pH 7	17.6ml
10% sarcosyl	26.4ml

The above reagents were dissolved in the manufacturer's bottle at 65° C and the solution stored in the fridge.

For use, Soln. D was prepared by adding 72 ul of β-ME to 10ml Soln. D.

Tris saturation of phenol

Stocks of 0.1 M Tris and 1 M Tris both DEPC treated and adjusted to pH 8 were prepared. To minimise handling of phenol, the bottle was thawed at room temperature and then melted at 68°C. Hydroxyquinoline was added (to prevent oxidation of phenol) to a final volume of 0.1% (w/v) and the solution transferred to a large conical flask with a side arm. An equal volume of 1 M Tris was added and the mixture was shaken and left to separate into two layers for 1 h or longer. The aqueous top layer was decanted and discarded using the side arm and an equal volume of 0.1 M Tris added to the flask and the process repeated with the weaker Tris solution until the phenolic layer reached a pH of greater than 7.8.

The phenol was then transferred back into the manufacturers bottle and stored in the fridge under 0.1 M Tris.

Extraction of RNA

1 ml of Soln. D was added to the cell layer which was subsequently scraped using a cell lifter, and the contents transferred to an eppendorf tube. 50 ul of 2 M sodium acetate pH 4 (helps to precipitate low molecular weight mRNA species), 500 ul of tris saturated phenol (see above) and 100 ul of chloroform - isoamyl alcohol (49:1) (the two solvents result in a better separation than one; also isoamyl alcohol improves the interface between the layers as well as decreasing foaming) were sequentially added with mixing by inversion in between each addition. The solution of RNA/phenol/chloroform was then shaken vigorously for 10 seconds and cooled on ice for 15 minutes. The samples were centrifuged at 13,000 rpm in a microfuge for 10 minutes. The aqueous top layer containing RNA was transferred into a fresh eppendorf tube and an equal volume of chloroform - isoamyl alcohol added. This was shaken vigorously and re-centrifuged for 5minutes. A second chloroform extraction was performed in the same manner and the aqueous layer was transferred to a fresh eppendorf tube. The RNA was precipitated by addition of an equal volume of isopropanol and storage at -20°C for at least 3 h.

Loading solution

This helps to keep RNA samples denatured.

60 ul of 10x MOPS (see below)

300 ul formamide

100 ul formaldehyde

140 ul DEPC water

Preparation of a formaldehyde gel

The gel was made up by first assessing the size of the hybridising species expected. Low % agarose separate out heavy molecular weight species to a better resolution. For a 1% (w/v) agarose gel, 30 mls 10 x MOPS buffer, 3 g agarose and 227 ml DEPC treated water were heated up until the agarose was fully dissolved. The solution was transferred to a fume cupboard and 40 ml of formaldehyde (to help keep RNA samples denatured) was added and the solution cooled slightly before pouring into a gel mould containing a comb to form wells in a levelled gel tank in the fume cupboard. After the gel had set, the tank was filled with 1x MOPS buffer before removing the comb. The samples in loading buffer were added and electrophoresed.

10X MOPS (3(N-Morpholino)propanesulfonic acid)

200 mM MOPS

50 mM sodium acetate

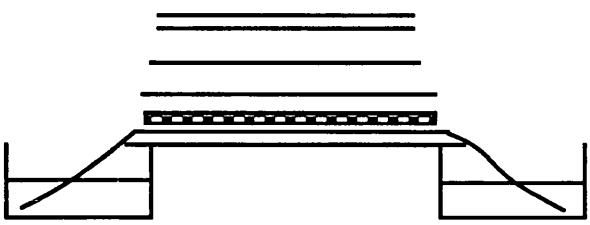
10 mM EDTA (ethylenediaminetetraacetic acid)

The reagents were dissolved in distilled water and the solution was adjusted to pH 7 with NaOH.

Ethidium bromide staining of a formaldehyde gel

The lanes for ethidium bromide staining were cut off and placed in 100 ml distilled water containing 10 ul ethidium bromide (10 mg/ml) for 30 minutes. The gel was subsequently destained in fresh water for at least 1 h and then placed on a UV transilluminator. Length of the two ribosomal bands from the origin (well) was noted.

Setting up a Northern blot



Cling film was placed around the edges of the gel to ensure transfer of SSC through the gel before the Hybond N+ membrane was carefully layed down. No air bubbles should be present between the membrane and gel, and lanes were carefully marked with pencil. A large weight was placed on top of the towels and the blot was left overnight.

20x SSC

350.6 g sodium chloride

176.4 g sodium citrate

distilled, deionized water 1.8 litres

Salts were dissolved in the water and the pH adjusted to pH 7 with HCl before making the solution up to 2 litres.

Solution A

50 % formaldehyde (deionized, ultra pure)

0.25 M sodium acetate pH 5.2 (pH with glacial acetic acid)

These were made up in DEPC treated water.

Prehybridisation solution - 50 mls

10 mls 50% dextran sulphate

10 mls 50 x Denharts (see below)

16.5 mls formamide

10 mls 10 x SSC (see below)

1.25 mls 20% SDS (lauryl sulphate)

200 ul 0.5M EDTA

0.5 ml denatured (100°C for 5 minutes) 10 mg/ml salmon sperm DNA (see below)

Denharts solution

2g of BSA was dissolved in 80 ml of distilled, deionized (dd) water and the pH adjusted to pH 3 with 2 M HCl. The solution was placed in a boiling water bath for 15 minutes, cooled on ice for 10 minutes, adjusted to pH 7.5 with 2 M NaOH on ice and made up to 100 ml with ddwater. Meanwhile 100 ml of 2% (w/v) polyvinylpyrrolidone (PVP), 2% (w/v) Ficol 400 was prepared, autoclaved and cooled. The two 100 ml solutions were then mixed and frozen in aliquots.

Salmon sperm DNA (ss DNA)

1 g of ss DNA was added to 100 ml ddwater, dissolved by warming and then sonicated and stored in eppendorf tubes in the freezer. Upon thawing, the DNA was syringed to make the solution less viscous, denatured at 100°C for 5 minutes and added to the prehybridisation mixture.

10 X SSC

175.3 g sodium chloride (molecular biology grade)

88.2 g sodium citrate (molecular biology grade)

The salts were dissolved in 1.8 litres of DEPC treated water and the pH adjusted with HCl to pH 7. The volume was made up to 2 litres and then filtered through a 0.45 u millipore filter into a sterile bottle.

Incorporation of radiolabelled nucleotides

This was performed by spotting two lots of 1 ul of each probe onto Whatman DE81 ion exchange paper and washing one of the pieces of Whatman paper in $NaHPO_4$ (3 x for 5 minutes), water (1 x for 2 minutes) and absolute ethanol (1 x for 1 minute). The washed paper was allowed to dry and the Whatman discs were placed in vials containing optiphase 'hisafe' scintillant and counting on the Beta counter. Incorporation of nucleotides was then calculated by dividing the washed filter counts by the unwashed filter counts.

CHAPTER 5

LOCALISATION OF IL-6 PROTEIN AND mRNA BY IMMUNOCYTOCHEMICAL STAINING AND *IN SITU* HYBRIDISATION.

ABSTRACT

It has been demonstrated in the previous chapter that bioactive IL-6 protein and mRNA for IL-6 is produced by human osteoblast-like cells *in vitro*. In order to obtain information about the expression and role of IL-6 *in situ*, immunolocalisation and *in situ* hybridisation techniques were studied using cryostat cut tissue sections of bone and tumour.

Immunolocalisation techniques in tissue sections were confounded by high non-specific binding and several attempts were made to reduce this. Cells cultured on multispot slides provided more satisfactory results. IL-6 was localised in osteoblast-like cells treated with monensin. Furthermore co-localisation of IL-6 with a marker of osteoblast phenotype, alkaline phosphatase, was demonstrated in these osteoblasts. IL-6 protein was present in nearly all the cells whereas alkaline phosphatase was only present in a proportion of the cells. IL-6 was also localised in osteoclasts derived from an osteoclastoma and was present in all the osteoclasts although intensity of staining was variable. By pre-incubating IL-6 with the anti-IL-6 antibody before addition to the multispot slides it could be demonstrated that the IL-6 antibody was specific for IL-6.

Several different tissue sections were used in the *in situ* hybridisation study which comprised of osteophytic osteoarthritc bone, giant cell tumor, and fetal tissue.

RNA probes were used for *in situ* hybridisation. The IL-6 cDNA insert was present within a vector containing only one bacterial promoter and so it was necessary to subclone the insert into a vector containing two promoters in order to obtain both sense and anti-sense RNA probes.

IL-6 mRNA was localised in many cells within the osteophytic sections including osteoblasts, osteoclasts, osteocytes, marrow cells and chondrocytes. Similar areas of tissue were stained for tartrate resistant acid phosphatase (marker of osteoclast phenotype). By using this stain as well as a Wright's stain, the osteoclasts were easily identified in the sections containing RNA probes.

This method has the potential to provide valuable information about cells and their function within the remodelling cycle. The expression of IL-6 by osteoclasts raises interesting questions about the functions of this cell in bone remodelling in that it may not play a purely resorptive role. It is possible that these multinucleated cells also have a communicative function in the remodelling cycle.

INTRODUCTION

Immunohistochemistry is a useful tool to study cytokine expression, but at any one time insufficient protein may be available for positive detection. In addition, if the protein is detected, it may have arrived there by some other route such as endocytosis, entrapment or special transport systems rather than by de novo synthesis. Only *in situ* hybridisation of mRNA unequivocally identifies the protein producing cells *in vivo*. The localisation of a particular mRNA within a cell could indicate which cells are transcriptionally active although this does not mean that the cell will translate the mRNA into the final mature protein (McDonald and Tuan 1989). Therefore it is preferable to study both immunolocalisation and *in situ* hybridisation techniques in parallel.

Using immunofluorescence, Andersson and Matsuda (1989) successfully localised IL-6 protein in peripheral blood mononuclear cells. This procedure utilized a murine specific anti IL-6 antibody bound to an FITC-coupled goat anti mouse immunoglobulin. Tabibzadeh et al (1989) also demonstrated specific IL-6 immunoreactivity in human tumors using an avidin-biotin-peroxidase detection

system. Furthermore preadsorbtion of IL-6 antiserum with IL-6 antigen blocked specific staining in the sections Tabibzadeh et al (1989).

Several groups have employed the technique of *in situ* hybridisation to localize specific mRNA sequences within various tissues. There seems to be a number of contentious points when studying this technique, the first of these being the choice of probe. Single stranded RNA probes have several advantages over double stranded DNA probes. Using anti-sense RNA probes, there is a large increase in sensitivity due to the absence of self-reassociation which is a problem associated with double stranded cDNA probes. RNA-RNA duplexes, formed when the RNA probe hybridises with the particular mRNA, have a higher stability than RNA-DNA hybrids. RNA probes characteristically exhibit less non-specific binding than DNA probes. The generation of sense and anti-sense RNA probes results in the production of transcripts of similar concentration and fragment length. Furthermore, by adjusting the volume of the sense and anti-sense probes, equal amounts of ^{35}S radioactive counts are obtained for both conditions. The sense strand should be identical to the mRNA produced in the cell and no binding should occur; therefore an appropriate negative control is obtained. Another advantage of using RNA probes is evident during the washing procedure where RNase A is used to digest unbound and non specifically bound RNA probes. (The probe-target hybrids are resistant to this digestion.) The major disadvantage of RNA probes is the sensitivity to digestion due to naturally occuring RNases prior to hybridisation. This makes handling and treatment of the tissue and probes more difficult (Ogilvie et al 1990).

In situ hybridisation at first utilized radioactive probes (Bratic and Hoase 1978, Angerer and Angerer 1981, Lawrence and Singer 1986, Croen et al 1987, Sandberg and Vuorio 1987, Lieure et al 1989, Bosseloir et al 1989, Kato et al 1990a, Noji et al 1990, Ogilvie et al 1990, Furuta et al 1990). ^{35}S was found to be

the most appropriate isotope which provided a good balance between activity and resolution as opposed to ^{3}H and ^{32}P. The disadvantages of radioactively labelled probes are that they are time consuming, expensive and not easily quantitated. Non-radioactive probes can provide a quicker, quantitative method although they are not as sensitive. Biotinylated probes are the best studied non-radioactive method (Burns et al 1985, Lawrence and Singer 1986, McDonald and Tuan 1989, Pringle et al 1989, Furuta et al 1990, Morris et al 1990). These could be detected in a number of ways such as avidin-biotin, avidin-fluorescein or by antibodies to biotin (Burns et al 1985). Alkaline phosphatase or peroxidase conjugated to streptavidin-biotin was then used to amplify the signal. Burns et al (1985) used a further amplification which results in the deposition of silver and was found to be a good sensitive method in their hands. The major problem with biotinylated probes is the lack of sensitivity and high non-specific binding (Morris et al 1990, Ogilvie et al 1990). More recently digoxigenin, a derivative of cardiac glycoside digoxin has been utilized to tag probes. It does not appear to have the same specificity problem as biotin (Morris et al 1990, Furuta et al 1990), and appears to be almost as sensitive as ^{35}S labelled probes (Furuta et al 1990). The study presented in this study utilises ^{35}S labelled RNA probes as they are sensitive and appropriate sense controls are generated.

The next question addressing this technique is the use of an appropriate fixative to preserve intracellular mRNA and tissue morphology. Several groups have compared different fixatives (Pringle et al 1989, McDonald and Tuan 1989). The majority of workers successfully used cross-linking agents such as paraformaldehyde, glutaraldehyde and formalin (Angerer and Angerer 1981, Bosseloir et al 1989, Cox et al 1984, Pringle et al 1989, Kato et al 1990a, Furuta et al 1990).

Access of the probe to mRNA within the cells is a further consideration for successful hybridisation. Many groups employ demineralization and proteinase K treatments to break down large structures such as hydroxyapatite (Bratic and Hoase 1978, Angerer and Angerer 1981, Cox et al 1984, Croen et al 1987, Sandberg and Vuorio 1987, Pringle et al 1989, McDonald and Tuan 1989, Furuta et al 1990). Concentrations of proteinase K have been found to be critical for various tissues (Angerer and Angerer 1981, Pringle et al 1989) whereas other groups have demonstrated that this treatment is unnecessary (Ogilvie et al 1990). The use of triton-X-100 to permeabilize the sections has also been used by some workers (Bosseloir et al 1989, Morris et al 1990).

Size of probe also has some bearing on the effectiveness of this procedure; optimal size of RNA probes for *in situ* hybridisation is generally 50-250 bases (Ogilvie et al 1990). As the IL-6 RNA probe is approximately 300 base pairs, access of this probe was considered to be satisfactory for this technique.

Many groups also included a protein acetylation stage to reduce electrostatic binding of the probe to any remaining proteins (Angerer and Angerer 1981, Cox et al 1984, Croen et al 1987, Sandberg and Vuorio 1987, Kato et al 1990, Noji et al 1990). The content of the hybridisation mix also varies in different studies although the consensus favours 50% deionized formamide (Bratic and Hoase 1978, Cox et al 1984, Coghlan et al 1985, Burns et al 1985, Lawrence and Singer 1986, Croen et al 1987, Sandberg and Vuorio 1987, Bosseloir et al 1989, Pringle et al 1989, Furuta et al 1990, Kato et al 1990). Other important components include Denhardts solution which blocks hydrogen bonding of the probe to basic proteins, dextran sulphate which increases the hybridisation signal, tRNA and salmon sperm DNA to block non specific binding, and DTT when using ^{35}S labelled probes to prevent their oxidation.

Washing procedures also vary enormously although an RNase digestion stage is usually incorporated when RNA probes are used (Kato et al 1990, Noji et al 1990) to reduce non specific binding further.

In situ hybridisation has been used to successfully localise IL-6 mRNA in human tonsils (Bosselior et al 1989), mononuclear cells (Kato et al 1990) and rheumatoid synovium (Ogilvie et al 1990).

The study presented in this chapter investigates the localisation of IL-6 mRNA in various tissues by *in situ* hybridisation. Osteoarthritic osteophytic bone was used for this technique, especially at the initial stages, as it was a very cellular tissue containing all stages of bone remodelling. Once the conditions were optimised for the IL-6 RNA probe, other tissues were investigated: namely osteoclastoma tumours and fetal bone.

METHODS

Immunolocalisation - Staining

Human osteoblast-like cells were trypsinised, resuspended in EMEM + 10% (v/v) FCS and 3000-5000 cells in a 30 ul volume were plated out on 10 spot slides and left to adhere for 24 h. Following this, 30 ul of $1,25(OH)_2D_3$ was added in EMEM + 10% (v/v) FCS (to stimulate alkaline phosphatase) for 24 h. Stimulatory agents for IL-6 (based on bioassay results) were subsequently added in the presence of 1 uM monensin, which blocked secretion of proteins from cells, for 6 h. The cells were fixed by addition of 4% paraformaldehyde, 1% $CaCl_2$ in water pH 7 for 15 minutes at room temperature and were left to dry and stored at -70°C wrapped in aluminium foil.

Osteoclasts were obtained from giant cell tumours. The tumour was roughly chopped up in EMEM and wetted sections placed on 10 spot slides. Some of the

osteoclasts tended to settle on the glass slides and they were subsequently fixed in acetone for 10 minutes then left to air dry before freezing.

Sections of bone, tumour and osteophytes were placed on 4 spot slides and fixed in acetone as for the osteoclasts.

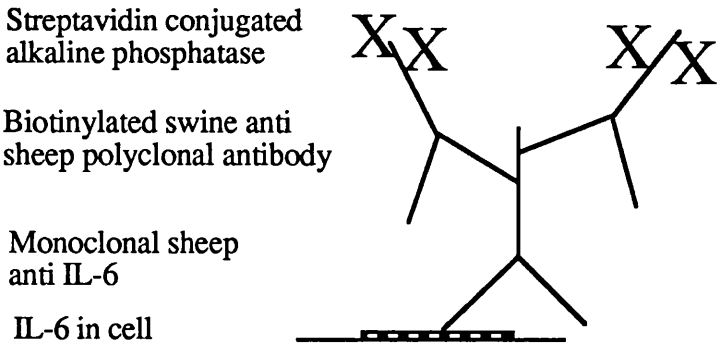
Alkaline phosphatase staining

10 mg of Napthol AS MX phosphate was dissolved in 0.5 ml ethylene glycol monoethyl ether EGMEE. 20 mg of fast blue BB dye was added and the solution was mixed. This was then added to 50 ml of 0.1 M Tris pH 9 - 9.2 and the solution stirred and filtered onto the slides for staining. The colour was left to develop in the dark for up to 30 minutes.

The slides could then be co-localised for a particular cytokine such as IL-6. In this case, $1,25(OH)_2D_3$ (10^{-8} M) was incubated with the human osteoblast-like cells for 24 h in order to induce their differentiation. IL-1 (10^{-11} M) and monensin (1 uM) were subsequently added for a further 6 h to stimulate IL-6 expression and the cells were fixed and stored as described previously.

IL-6 staining

Principal



This method is used to amplify a signal. It is especially useful when only small quantities of protein are present. The primary antibody is sandwiched between the antigen and a biotinylated polyclonal antibody raised against the primary antibody

species. The biotin moiety subsequently binds to streptavidin conjugated to alkaline phosphatase and using a fast red salt and Napthol phosphate, a red product can be detected.

The slides, containing cells or sections, were placed in tris buffered saline (TBS) (0.05 M Tris/HCl, pH 7.6, 0.15M NaCl) for 5 minutes. Phosphate buffers should not be used as phosphate inhibits the alkaline phosphatase reaction. Excess liquid was drained off the slides and using a tissue the edges were carefully wiped between the spots. The sections or cells were incubated for 20 minutes with normal serum from the secondary polyclonal antibody species (eg swine) diluted 1:5 in TBS. During all incubation stages, the slides were placed in a humidified box. The excess serum was then tapped off and primary antibody optimally diluted in TBS was incubated with the cells for 20 - 30 minutes. As a negative control, half of the cells or sections were incubated with normal serum from the primary antibody species diluted to the same extent as the antibody (eg sheep serum). In between each stage, the slides were gently washed in TBS using a pipette, taking great care to keep all the different treatments separate, and subsequently immersed in a TBS bath for 5 minutes. Biotinylated antibody, followed by streptavidin/alkaline phosphatase, optimally diluted in TBS were sequentially added to the wiped slides for 30 minutes. After the final wash, the slides were incubated for 10 - 15 minutes with alkaline phosphatase substrate solution (see Appendix 4).

The slides were subsequently washed with distilled water and counterstained with haematoxylin for 20 seconds. The counterstain was washed off with water and the slides were dried and mounting fluid (90% glycerol, 10% PBS) was added. A glass coverslip was carefully layered over the slide and the edge sealed with clear nailvarnish. Once dried, the sections and cells could be photographed.

In situ hybridisation

Tissue sections were cut using a cryostat and mounted on TESPA coated (See Appendix 4) 4 spot slides. All equipment and solutions were RNase free by handling with gloves and treatment with DEPC (see Chapter 4 methods). Sections were fixed in DEPC treated paraformaldehyde (4% in PBS) for 5 minutes followed by extensive washing in DEPC treated PBS, air drying and storage at -20°C in aluminium foil.

Cryostat sections were cut by Dr. R.A.Dodds (see acknowledgements).

Generating the RNA probes

Principal

The DNA insert is present in the polylinker site of the transcription vector (eg IL-6 in blue script). The promoters for T3 and T7 RNA polymerases are located on either side of the polylinker (area where there are multiple enzyme restriction sites). T3 and T7 RNA polymerases specifically transcribe DNA sequences downstream of the T3 or T7 promoters respectively. Cloned DNA inserts within the polylinker region are transcribed from either promoter. T3 and T7 RNA polymerase use the cloned DNA as a template and synthesize complementary RNA in the presence of Mg^{2+} and ribonucleoside triphosphates. Spermidine stimulates enzyme activity. Highly specific labelled transcripts are obtained when radiolabelled ribonucleoside triphosphates (eg ^{35}SCTP) are present in the reaction. The vector must be linearised with a suitable restriction enzyme before initiation of the transcription reaction in order to obtain transcripts of a defined length. Using intact plasmid DNA as a template for transcription will result in heterogeneous transcripts of multiple lengths. The DNA template may be removed by digestion with DNase 1, RNase-free, after the reaction. Ribonuclease inhibitor may be added to the transcription reactions to ensure the preservation of the full-length single-stranded RNA molecules.

Generation of RNA probes

The vector containing the insert of interest eg IL-6 was linearised using the two enzymes which cut at the junction between vector/insert. The two enzymes were reacted separately so that two different linear species were obtained (see Appendix 4). These were then purified using a 'gene clean kit' containing glass milk.

The RNA probes were made from the cDNA using an SP6 T7 T3 kit (Boeringer). About 500 ng of DNA cut with each enzyme was transferred to two eppendorf tubes containing 3 ul of ATP, GTP, UTP mix; 2 ul 10 X buffer; 1 ul RNase inhibitor, 2.5 ul ^{35}S CTP and 1 ul of the appropriate polymerase (either T7 or T3 for inserts contained within the blue script vector - see Appendix 4). The volume was adjusted to 20 ul with DEPC treated water and the tube placed at 37°C for 30 minutes. Transcripts were generated by the enzyme binding to the directional bacterial promoter and incorporating the complementary nucleotides for the insert cDNA sequence. When the enzyme reached the end of the sequence, the reaction was terminated and started again thereby generating a series of transcripts.

Following this, 2 ul of DNase 1 (RNase free) was added for 15 minutes at 37°C. The reaction was stopped with 2 ul of 0.5M EDTA (DEPC treated). Therefore the tubes contained RNA probes generated from cDNA templates. One enzyme produced a sequence complementary to the coding strand (anti-sense) and the other enzyme produced a sequence complementary to the non-coding strand (sense).

The volume was made up to 50 ul with DEPC treated water and incorporation of radiolabelled nucleotides assessed (see Appendix 3)

Spun columns were prepared using the outer part of a 1 ml syringe. A small plug of glass wool was pushed into the bottom of the syringe and Sephadex G25 (suspended in ddwater and pre-expanded in a 90°C water bath for 1 h followed by suspension in DEPC treated water) was carefully layered into the syringe avoiding

air bubbles. This creates a molecular sieve whereby the unincorporated trinucleosides were retained in holes in the Sephadex G25. The relatively large transcripts filter through the Sephadex G25 and are therefore separated out. Once the column was filled, it was placed in a 15 ml centrifuge tube and spun at 2000 rpm for 4 minutes. The water was then tipped out and an eppendorf tube (without lid) (RNase free) placed into the bottom of the 15 ml tube. The column was then carefully set up so that the tip protruded into the eppendorf tube. The appropriate probe was then added to the top of the column carefully. This was then spun at 2000 for 4 minutes. During this time the probe was collected in the eppendorf tube which was then capped and frozen ready for use.

Prehybridisation

All solutions were DEPC treated. The slides were dipped into a series of solutions at room temperature in DEPC pre-soaked coplin jars. These were: PBS (0.1 M pH 7.2) for 3 minutes, 0.1 M Glycine/PBS for 2 x 3 minutes, and PBS for 3 minutes. This rehydrated the cells and sections on the slides.

For the sections only, the slides were demineralized with citric acid buffer (100 ml of 1M citric acid (tri sodium salt) was adjusted to pH 4.5 with 1 M citric acid (free acid). This was then diluted 1:4 with ddwater) at room temperature for 1 h in order to break down large moieties such as hydroxyapatite and thereby reduce non specific binding and allowing access of the RNA probe to the mRNA species. This was followed by proteinase K treatment (1 ug/ml diluted in 0.1 M Tris (pH 8) 50 mM EDTA (pH 8)) at 37°C for 5 minutes (cells) or 20 minutes (sections). Proteinase K degrades proteins in order for the probe to gain access to the mRNA within the cells. The slides were then re fixed in 4% paraformaldehyde/PBS room temperature for 5 minutes in order to retain the cellular localisation of the mRNA before washing in PBS twice (3 minutes).

The remaining proteins were acetylated with acetic anhydride (0.5 ml/200 ml of 0.1 M triethanolamine). The anhydride is only active for 1 minute and so the solution was prepared just before addition to slides. This was left for 10 minutes before addition of 50% deionized formamide/2 X SSC for a further 10 minutes. (This pre- equilibrates the sections in organic phase).

Hybridisation

The formamide/SSC was carefully drained off and 10 - 15 ul of hybridisation buffer (see Appendix 4) was carefully delivered to each section.

This mixture helps to block non specific binding of the probe to the cells. tRNA interchelates with looped sequences within the DNA which would otherwise attract the probe. The function of the other components is discussed in the introduction of this chapter. The slides were placed in a humidified (DEPC treated 2 X SSC) box in a 42°C waterbath for at least 3 h (tissue paper was taped to the inside of the lid to prevent drops of liquid splashing onto the slides).

Without creating air bubbles; 5 - 10 ul of each RNA probe was added (diluted in more hybridisation buffer) to each section so that the specific activity of the RNA probe was 300,000 cpm per section. The slides were then covered carefully with parafilm strips to prevent evaporation and care was taken to ensure that the different RNA probe conditions remained separate.

The slides were incubated in a humidified box overnight at 42°C.

Washing

The parafilm was carefully removed from the slides at room temperature in a coplin jar containing 4 X SSC and left for 30 minutes. Four 15 minute washes in 4 X SSC were performed in a shaking water bath set at 37°C. The slides were washed in a solution of 0.5 M NaCl/10 mM Tris-HCl pH 8/ 1 mM EDTA for 10 minutes at 37°C and were subjected to RNase digestion in the previous solution containing 20

ug RNase A/ ml for 30 minutes at 37°C. The slides were washed in 2 X SSC for 30 minutes at 37°C followed by a higher stringency wash of 0.1 X SSC for 30 minutes at 37°C. The cells were progressively dehydrated in 70% ethanol/0.3 M ammonium acetate for 5 minutes, 95% ethanol/0.3 M ammonium acetate for 5 minutes and twice in 100% ethanol/0.3 M ammonium acetate for 5 minutes. The sections were air dried, placed in a box containing dessicant packets and stored at -20°C ready for autoradiography.

Autoradiography

LM1 emulsion was used to expose the radioactivity. At this stage, all work was performed in a dark room. The emulsion was pre-warmed in a coplin jar in a 45°C water bath (as the solid emulsion melted, air bubbles were removed using a needle). The slides were allowed to warm to room temperature before removing from the box. The sections were dipped into the emulsion and left for 5 seconds before smoothly drawing the slide out and allowing the excess emulsion to drain off. The slides were placed on an inverted metal tray on ice for 10 minutes (this helps to gel the emulsion so that it does not run off the slide whilst air drying). Following this, the slides were stood up in a rack and allowed to air dry. The dry slides were transferred to a box containing dessicant. The box was carefully wrapped in aluminium foil and by two black light proof bags and taped up. The box was placed at 4°C until development 2 weeks later.

Development

In the dark room, the slides were removed and placed in developer (Ilford phenisol high contrast developer) (diluted 1:4 in water) for 10 minutes. The slides were transferred to a 'stop' solution of 0.5 M acetic acid followed by 30% (w/v) sodium thiosulphate fixer for 10 minutes. Following this, the slides were removed from the dark room and extensively washed in several fresh changes of water before

staining with hematoxylin. The slides were air dried and coverslips placed over the sections using DPX mountant.

Subcloning IL-6 into Bluescript vector

IL-6 was present in a vector containing only one bacterial promoter. In order to obtain both a sense and anti-sense transcript, it was necessary to subclone IL-6 into another vector containing two bacterial promoters. Blue script vector was chosen as it contains T3 and T7 promoters and part of the β-galactosidase gene. This vector when transformed into XL-1 blue bacteria, forms blue or white colonies depending upon whether or not the insert has been ligated to the vector. The insert interupts the gene upon insertion into the polylinker region and the bacteria can not convert the substrate into a blue colour, therefore this is a simple selection procedure to isolate and amplify the insert.

IL-6 was isolated from the original vector by double digestion with Xba 1 and Sal 1. Several attempts to ligate the overhanging cohesive ends to blue script vector cut with the same enzymes failed. 100 ug of purified IL-6 insert cut with the above enzymes was subjected to a T4 DNA polymerase reaction in order to fill in the overhanging ends thereby creating blunt ends. This involved incubation of the DNA with enzyme and buffer which included nucleotides, for 30 minutes at 37°C (see Appendix 4). This was then phenol/chloroform extracted once and washed with chloroform before precipitation. In the meantime; bluescript vector (containing a polylinker region with many restriction enzyme sites) was digested with Sma 1 which results in blunt ends, and purified. A proportion of the vector and insert were then electrophoresed on a 1% (w/v) agarose gel along with a molecular weight marker. IL-6 insert is 300 bp whereas bluescript is 3000 bp long. Therefore 10 ng IL-6 contains more blunt ends than the equivalent amount of vector. This is important when setting up ligation reactions as an excess of vector is

required to account for the differences in size. From the gel, approximate amounts of DNA were estimated. It was considered that IL-6 amounted to about 10 ng of DNA whereas bluescript was approximately 100 ng. Therefore ligation mixes including stringent controls were as follows:

A) 3 ul IL-6, 1 ul vector, 1 ul ligase buffer, 1 ul T4 ligase enzyme and 4 ul water.

B) 6 ul IL-6, 1 ul vector, 1 ul ligase buffer, 1 ul T4 ligase enzyme and 1 ul water.

C) 1 ul vector, 1 ul ligase buffer, 1 ul T4 ligase enzyme and 7 ul water.

D) 1 ul vector, 9 ul water

E) 1 ul uncut vector, 9 ul water.

C - E were controls. C was the ligation control - theoretically, the ligase should have re-circularised the vector without disrupting the β-galactosidase and therefore many blue colonies should be apparent after transformation and plating out. This shows that the ligase enzyme is functional. D and E are to prove that the XL-1 blue bacteria are competent. The competent bacteria should take in the closed circular bluescript with at least 100 fold more efficiency than the unligated vector.

These ligations are blunt-ended and therefore the reaction is allowed to proceed over night at 16°C.

Half of the ligation mix for each reaction was removed and transformed into competent XL-1 blue bacteria (see general methods). The bacteria were spread onto ampicillin (50 ug/ml) containing agar plates which had previously been spread with substrate for the β-galactosidase reaction. This involved addition of 0.8 mg/ml of X-gal and 0.8 mg/ml isopropylthio-β-D-galactosidase (IPTG) to the plates after which the substrate was allowed to soak into the agar for about an hour prior to the addition of the bacteria. Once the bacteria were spread, the plates were inverted and placed in a 37°C incubator over night.

On the following day, there were more white colonies on A) and B) when compared to C) (30% compared to 10%). 10 colonies were picked and grown up in 5 mls L-broth + ampicillin over night (see Chapter 2). 1.5 mls was then removed from each preparation and spun down in an eppendorf tube. The resulting pellets were then subjected to small scale plasmid preparation (see Chapter 2). After quantification of the DNA (see Appendix 1), 2.5 ug was digested with a restriction enzyme with only one cutting site in the vector-insert sequence. This was electrophoresed on a 1% (w/v) agarose gel along with molecular weight markers. The insert, present within the vector, should be approximately 3.3 kb long (fig. 5.1a). Only one of the ten preparations did not contain the insert. This was checked by Southern blotting the gel onto Hybond N+ under denaturing conditions (0.4 M NaOH) for 1 h. The Hybond N+ was subsequently probed for IL-6 (see Chapter 4 for method). The filter was set up for autoradiography for 30 minutes (see fig. 5.1b). The probe annealed to all the preparations except the one which did not contain the insert. One of the preparations was chosen and grown up in 250 mls L-Broth + ampicillin overnight (from the remaining 3.5 mls). A large scale plasmid preparation was performed and the 300 bp insert checked by double digestion with Eco R1 and Xba 1. By restriction enzyme digests, the orientation of the insert and therefore which restriction enzyme resulted in the sense and anti-sense transcripts was ascertained. RNA probes could then be generated for IL-6.

RESULTS

Immunolocalisation

By doing a series of titration experiments, optimum IL-6 staining of cells was obtained with sheep anti-human IL-6 antibody at 1:100 dilution, biotinylated swine anti-sheep antibody at 1:200 dilution and streptavidin conjugated to alkaline phosphatase at 1:200 dilution (data not shown). Presence of IL-6 protein was

observed in both human osteoblasts and human osteoclasts (fig. 5.2a,b). Non-immune sheep serum at a dilution of 1:100 was used as a negative control (figs. 5.2c,d, and fig. 5.3b).

In a separate experiment, IL-6 was co-localised with a marker of the osteoblast phenotype, alkaline phosphatase. Alkaline phosphatase was present in a proportion (approx. 40%) of the cells which may indicate that osteoblasts at different maturation stages are present. Intracellular IL-6 staining was observed, using the sheep anti-human IL-6 antibody (1:100), in over 90% of the cells (fig. 5.3a).

In order to ascertain that the IL-6 staining was specific, the anti IL-6 antibody (1:100) was preadsorbed with varying concentrations of rhIL-6 prior to addition to the cells (fig. 5.4). Preadsorption of the anti IL-6 antibody with rhIL-6 at a concentration of 10,000 pg/ml resulted in partial inhibition of antibody interaction with endogenous IL-6 production by the human osteoblasts and osteoclasts (fig. 5.4c,d). A concentration of 500,000 pg/ml of IL-6 totally inhibited antibody binding to the osteoblasts and osteoclasts (fig. 5.4e,f).

The anti-IL-6 antibody was used for the immunolocalisation of IL-6 in various cryostat cut sections of tissue, including human fetal bone, osteoclastoma and osteophyte. Non specific binding was very high and various attempts were made to reduce this, such as pre-incubation with avidin and biotin, variation of secondary antibody species and increasing levamisole content in the substrate solution. Some of these conditions only reduced, but did not abolish the high non specific binding (data not shown).

In situ hybridisation

The developing osteophyte

Osteophytes are characteristic bony outgrowths that develop in osteoarthritic joints in the human (Hamerman 1989). This rapidly developing bone is an excellent tissue for *in situ* hybridisation study as it is highly cellular, contains all the stages of the remodelling cycle and exhibits both endochondral and intramembranous bone formation (see Chapter 1).

Endochondral bone formation

Fig. 5.5a. shows a histology of an osteophyte and demonstrates the process of endochondral bone formation. Osteophytes are composed of cancellous trabeculae covered by cartilage and fibrocartilage. The matrix around the hypertrophic chondrocytes calcifies in the preliminary stages of endochondral bone formation. Invasion by vascular connective tissue results in the mass of calcified cartilage. Osteoclastic resorption of this cartilage is followed by the deposition of woven bone on these eroded surfaces. In this way trabeculae of woven bone replace and eminate from the initial cartilage. In a similar process of remodelling, osteoclasts resorb this woven bone which is ultimately replaced with parallel fibred lamellar bone. During acute bone remodelling the marrow stroma is highly cellular (hematopoietic tissue) and is rich in mononuclear cells and migrating osteoclasts. Fig. 5.5b shows a higher magnification of a similar area of endochondral ossification. Anti-sense binding of the IL-6 RNA probe to the tissue was particularly intense in the osteoblasts although varying degrees of hybridisation were evident within these cells (Fig. 5.5b). Furthermore; IL-6 mRNA was only present in a population of chondrocytes, with the more hypertrophic chondrocytes lacking IL-6 mRNA expression. Marrow cells were also positive for IL-6 mRNA

expression. The binding was specific as the sense transcript exhibited no binding (fig. 5.5c).

Intramembranous ossification

The histology in fig. 5.6a demonstrates the deposition of woven bone by a fine layer of osteoblasts that have differentiated from the surrounding connective tissue. Similar to that observed in endochondral bone formation, the newly formed more rounded osteoblasts, actually laying down osteoid, expressed higher levels of IL-6 mRNA than the more mature osteoblasts (lining cells) apposed to the bone surface (fig. 5.6b). A similar situation is evident at the tip of the developing osteophyte, whereby both types of bone formation are observed within the same section. Fig. 5.7a illustrates a very early stage of endochondral bone formation; woven bone is formed by osteoblasts apposed to the cartilage. Concurrently, large osteoblasts differentiate from the surrounding connective tissue and either form woven bone directly (intramembranous) or congregate and appose distinct areas of the newly formed cartilage. High IL-6 mRNA expression was observed in the rounded newly formed osteoblasts (fig. 5.7b); this was absent in the corresponding negative control (fig. 5.7c).

Maturation of the osteophyte

Fig. 5.8a is a histology of another section of developing osteophyte depicting mature lamellar bone and a spicule of newly formed bone lined by osteoblasts, surrounded by connective tissue and a small pocket of marrow. Osteoblasts, osteocytes, marrow cells and a population of the chondrocytes all hybridised positively with the IL-6 anti-sense probe (fig. 5.8b). In addition some larger cells, possibly osteoclasts within the marrow, also expressed IL-6 mRNA. Hybridisation was specific as the sense probe exhibited no binding (fig. 5.8c).

IL-6 mRNA expression by osteoclasts

Fig. 5.9a shows the presence of osteoclasts stained for TRAP activity in an area of endochondral bone formation. Fig. 5.9b shows osteoclasts in a similar area which are expressing very high levels of IL-6 mRNA. Figs. 5.10a and 5.10b demonstrate IL-6 anti-sense hybridisation to other osteoclasts within different osteophytes.

Human osteoclastoma

Osteoclastoma is a rare form of tumour, often not malignant and usually found in the shaft of long bones. This tumour is characterised by high numbers of giant cells, although it is the stromal cells that form the neoplastic element (Goldring et al 1987). These giant cells have been shown to possess several characteristics of osteoclasts such as their ability to resorb bone slices *in vitro* (Horton et al., 1985, James et al., 1991) and retraction of cytoplasmic processes upon addition of calcitonin (Horton et al, 1985). Some unknown element appears to promote differentiation of osteoclast formation, as the percentage of osteoclasts within the tumour compared to that in bone is extremely high. Within the tumour, areas of active bone formation can be observed. IL-6 mRNA expression in the osteoclastoma was very intense and specific to the anti-sense probe (fig. 5.11a) as the sense RNA probe exhibited no binding (fig. 5.11b). The anti-sense probe hybridised to all the cells within tumour including the stromal cells and osteoclasts (Fig. 5.11a). Similar areas of tumour were reacted for TRAP activity (fig. 5.11c) and Wrights stain (histology) (fig. 5.11d).

Human fetal bone

Histology (fig. 5.12a) of a fetal rib section showed cartilage cells and connective tissue. Osteoblasts, derived from the connective tissue by the process of

intramembranous bone formation, are laying down immature woven bone in a strut-like fashion. The anti-sense IL-6 probe hybridised to the newly formed osteoblasts with a very intense signal similar to the osteophyte sections (fig. 5.12b). Furthermore, more flattened osteoblasts within the same section, although positive, did not express as much IL-6 mRNA. Other cells within the tissue were negative for IL-6 mRNA, and the sense transcript exhibited no binding (fig. 5.12c).

DISCUSSION

In chapter 4, the production of IL-6 by bioassay and Northern and dot blot analysis was demonstrated in human osteoblast-like cells. Furthermore, by employing immunostaining techniques in the present chapter, IL-6 protein was shown to be present in cultured osteoblasts. As IL-6 is expressed in such high levels in osteoblasts, and such a large proportion of cells are expressing the protein, it is possible to visualise that the cells in culture exist in a state where IL-6 mRNA and protein production are activated permanently. Culture conditions such as adherence to plastic or glass may be affecting the phenotype of the cells which results in an inaccurate reflection of the *in vivo* conditions.

Attempts to immunolocalise IL-6 in tissue sections were hindered by high non specific background. Many attempts were made to reduce this non specific binding, such as increasing levamisole content in the substrate solution; changing the species of the secondary antibody and pre-blocking solution, and pre-incubation with avidin and biotin. Although some of these treatments lowered the background slightly they did not abolish non specific binding. However, omitting a single step from the staining process resulted in an absence of binding which suggests that the negative control is inappropriate and is likely to be responsible for the non specific binding. Tissue sections contain proteins which may be interfering with the staining process by binding the non immune sheep serum which was used as a

negative control. Therefore studies are required using the specific isotype of the sheep anti-human IL-6 antibody for the negative control. Non-immune sheep serum is a poor negative control as it contains many of the immunoglobulin subclasses as well as serum associated proteins which may be interfering with the specificity of this procedure.

In situ hybridisation in developing osteophyte demonstrated that IL-6 mRNA was particularly intense in osteoblasts and osteoclasts. The anti-sense probe also hybridised to other regions in this tissue such as the marrow and a population of chondrocytes.

A striking feature of the *in situ* hybridisation studies was the high expression of IL-6 mRNA in what appears to be newly formed osteoblasts in both the osteophyte and the fetal tissue. It is very difficult to identify osteoblast progenitors *in situ*, because as yet there are no specific markers for these cells. Therefore histological examination and location of the cells are of critical importance for identification. More quiescent osteoblasts lining the bone surface express less IL-6 mRNA activity than plump newly formed osteoblasts and this was evident during both endochondral and intramembranous bone formation. Therefore, it appears that levels of IL-6 mRNA fluctuate within the osteoblasts. Further studies are required to investigate the co-expression of IL-6 mRNA with markers of osteoblast maturation. Several groups have demonstrated that proteins such as MGP, alkaline phosphatase, collagen type I and osteocalcin are expresssed at different stages of osteoblast maturation (Fraser et al 1988, Owen et al 1991, Lian et al 1991). Therefore the co-localisation of these proteins with IL-6 may possibly indicate whether IL-6 mRNA expression is a function of immature osteoblast phenotype.

IL-6 mRNA and protein were also localised in osteoclasts derived from osteoclastoma tumours. An advantage of using the osteoclastoma tumour for *in situ* hybridisation studies was the fact that negatives (sense RNA probe) were readily

obtainable. This is in contrast to osteophyte serial sections where the corresponding osteoclast was unlikely to be present in both anti-sense and sense labelled sections. Similar areas of sections were stained for TRAP activity and histologies were also performed in order to aid with the identification of the osteoclasts. In accordance with these studies, Ohsaki et al (1991) reported preliminary evidence for the presence of IL-6 mRNA in osteoclasts derived from osteoclastoma tumour. In a contrasting study, Hoyland et al (1992) demonstrated that osteoclasts did not produce IL-6 mRNA but were capable of synthesizing TGFβ mRNA. The results of Hoyland and co-workers (1992) were also in contrast to parallel studies within our laboratories by Merry et al (1991), who demonstrated that TGFβ and IL-1 mRNA were not expressed in osteoclasts (Merry et al 1991b). TNFα and TNFβ have been reported to be expressed in 14 day GM-CFC-derived osteoclasts (Qi et al 1991). Hence from the data presented within this chapter and evidence by other groups, it is likely that osteoclasts are not solely involved in resorption, but are probably involved in paracrine/autocrine interactions within the bone microenvironment. This is made possible by the expression of various cytokines by these osteoclasts. The ability of cells of the osteoclast lineage to synthesize factors implicated in local bone remodelling raises the possibility that osteoclastic cells could regulate other cells involved in bone turnover. As osteoclasts are difficult to isolate for *in vitro* study, and their phenotype is unstable under tissue culture conditions, the *in situ* hybridisation technique will be a valuable tool for investigating osteoclast function.

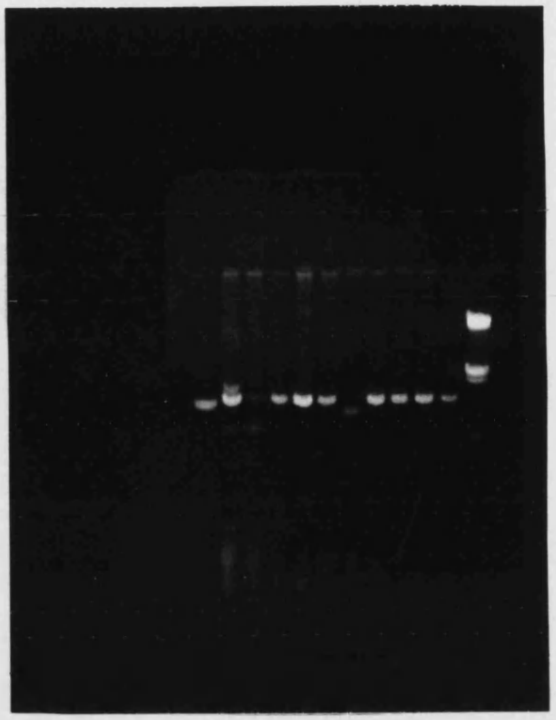
As IL-6 is produced by many of the cells within the bone microenvironment, it is less likely to be involved directly in bone formation and resorption in contrast to cytokines such as IL-1. The effects of IL-1 on bone remodelling are very potent and rapid. In accordance with this concept, studies within our laboratory have demonstrated that compared to IL-6 mRNA expression (Littlewood et al 1991b),

the expression of IL-1 mRNA is relatively transient (Merry et al 1992a) in human osteoblasts. However, the possibility that IL-6 may modulate actions of cytokines such as IL-1 can not be excluded. Indeed Black et al (1991b) has reported that the presence of IL-6 is critical for resorption in neonatal mouse calvariae stimulated by IL-1 and TNF, although others have been unable to demonstrate this same effect in a similar system (Al-Humidan et al 1991).

Conversely, it is possible that IL-6 expression within the bone microenvironment may need to reach threshold levels in order to exert any influence on bone remodelling and fluctuating levels of this cytokine in osteoblasts may be critical. In this respect, presence of inhibitors is also of importance when considering available IL-6 concentration. So far, no inhibitors for IL-6 have been isolated. This is in contrast to the functionally related cytokines TNF and IL-1. TNF inhibitors are binding proteins that associate with this cytokine and subsequently inhibit TNF function (Nophar et al 1990). In contrast, IL-1 inhibitors are receptor antagonist proteins, thereby blocking the association of the ligand with the receptor (Seckinger et al 1987a). α2-macroglobulin in the circulation (Matsuda et al 1989), and a soluble extracellular form of the IL-6R in urine (Novick et al 1989) have been identified as carriers for IL-6 although neither of these carriers inhibit IL-6 activity. So far, no natural soluble form of the IL-6R has been isolated in the circulation. Others, by using this truncated soluble IL-6 receptor, and by preparing specific antibodies, have established an ELISA (enzyme linked immuno-sorbant assay) (Yasukawa et al 1990, Saito et al 1991). This ELISA, although established in the murine system, may be useful for the detection of natural forms of the soluble IL-6R in the circulation. These potential carriers for IL-6 may protect the IL-6 from proteolytic breakdown and this could result in an increased local concentration of IL-6. Therefore, it is necessary to investigate whether inhibitors or carrier proteins for IL-6 exist within bone as these may influence the actions of this cytokine.

Further *in situ* hybridisation studies will be of value in order to gain a clearer understanding of the function of IL-6 in bone remodelling.

A



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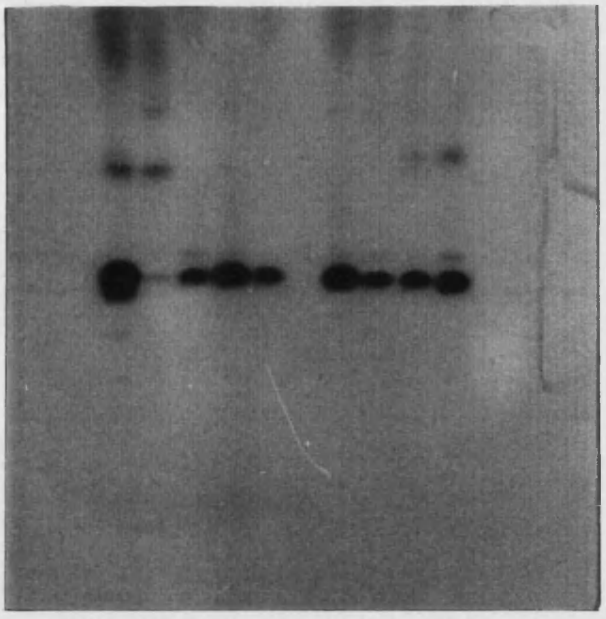
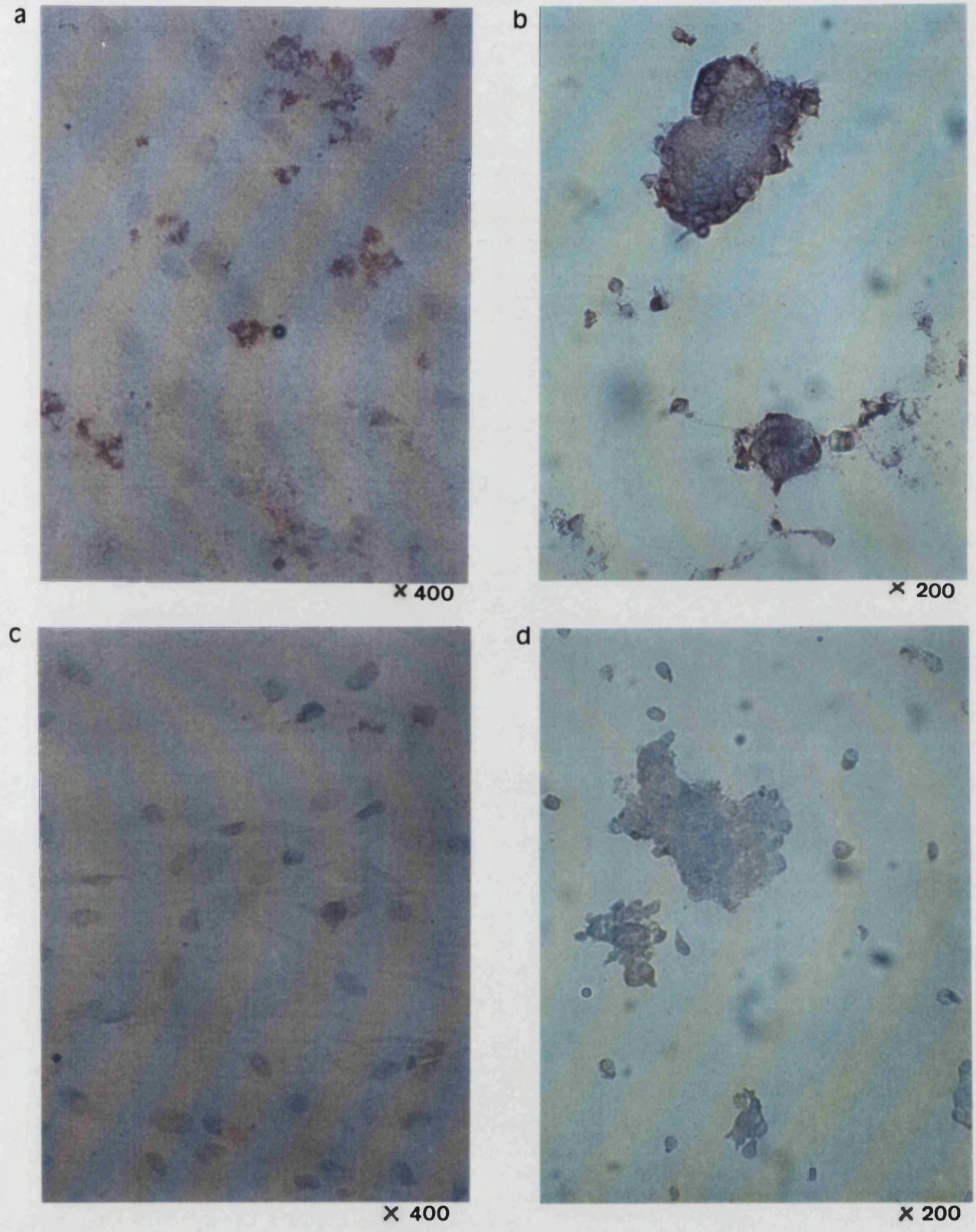


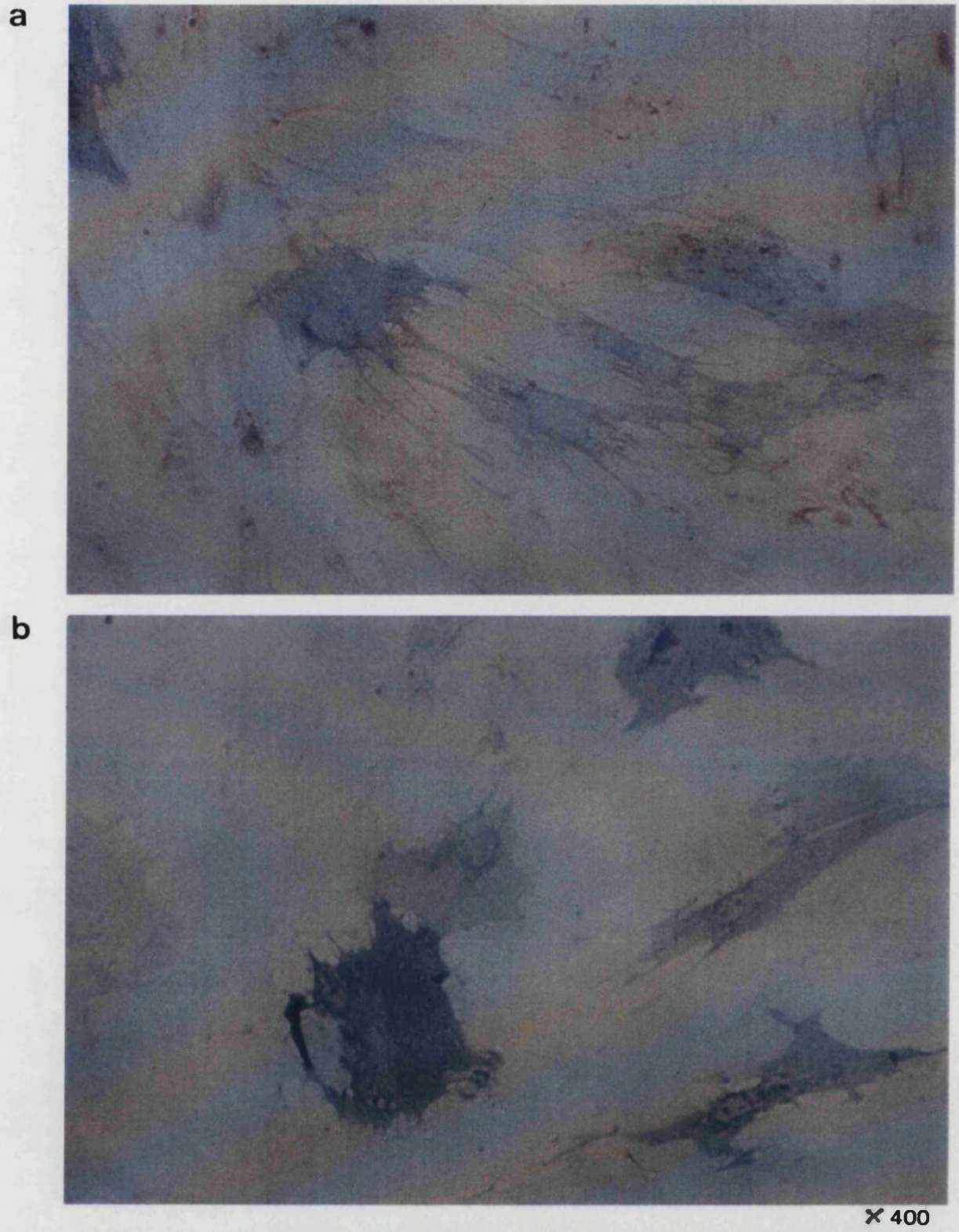
Fig. 5.1a - Ten small scale plasmid preparations (IL-6 300 base insert within 3000 base blue script vector) were linearised by digestion with Eco R1, and electrophoresed on a 1% (w/v) agarose gel (see Chapter 2). Lane 1 is the linearised vector alone and lane 12 is a molecular weight marker -λDNA cut with Hind III. Apart from sample number 6 (lane 7), all the linearised species appear to be larger than the 3000 base blue script vector alone.
Fig. 5.1b - The gel in fig. 5.1a was subjected to Southern blot onto a Hybond N+ membrane which was subsequently hybridised with an IL-6 cDNA probe (see Chapter 4 methods). All samples, apart from number 6 had incorporated the IL-6 insert as shown by the autoradiograph.

Fig. 5.2 Immunolocalisation of IL-6 protein in human osteoblasts and human osteoclasts.



Cells were immunostained with the sheep anti-human IL-6 antibody (see methods)
Fig. 5.2a IL-6 staining in human osteoblasts treated with monensin (1 uM) (see methods).
Fig. 5.2b IL-6 protein is present in human osteoclasts derived from an osteoclastoma.
Fig. 5.2c and 5.2d Negative controls - non immune sheep serum was incubated with the cells at the same concentration as the sheep anti-human IL-6 antibody.

Fig. 5.3 Colocalisation of IL-6 protein with a marker of osteoblast phenotype - alkaline phosphatase.



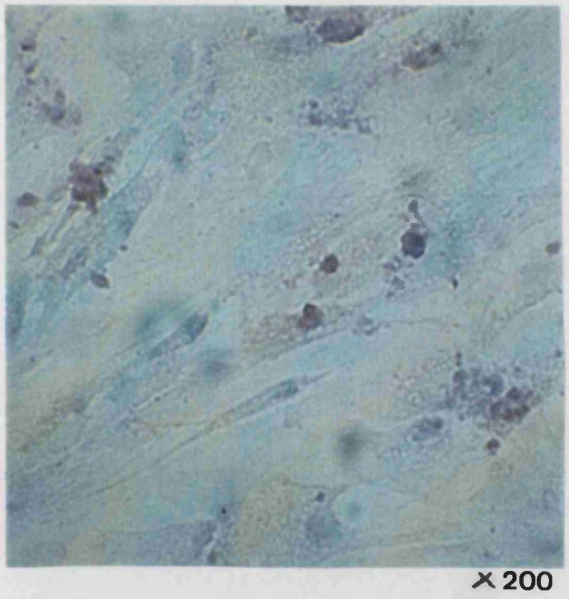
Human osteoblast-like cells were reacted for alkaline phosphatase (see methods) and subsequently stained for IL-6 protein by using the sheep anti-human IL-6 antibody (a). Non immune sheep serum was used as the negative control (b).

Fig. 5.4 Specificity of the sheep anti-human IL-6 antibody.

Fig. 5.4a and b IL-6 staining in osteoblasts (a) and osteoclasts (b) with sheep anti-human IL-6 antibody (1:100).

Fig. 5.4c and d Preadsorption of the anti-IL-6 antibody with 10,000 pg/ml of rhIL-6 in order to demonstrate partial inhibition of immunostaining in osteoblasts (c) and osteoclasts (d).

Fig. 5.4e and f Total inhibition of staining by preadsorption of the antibody with 500,000 pg/ml of rhIL-6 in osteoblasts (e) and osteoclasts (f) (compare with non immune sheep serum treated cells in Fig. 5.2c and d.


A
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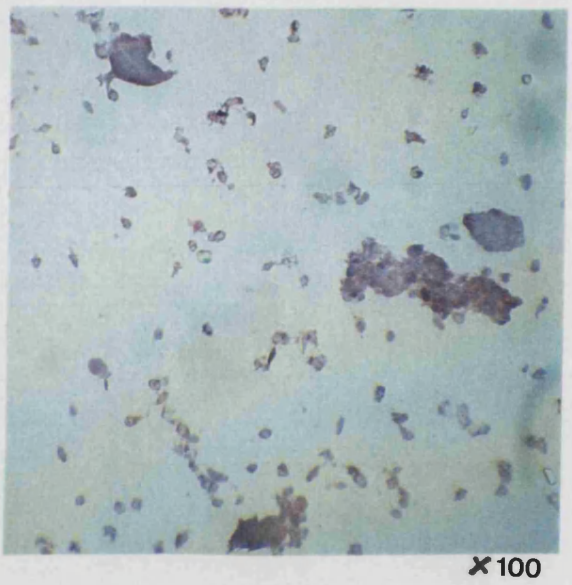
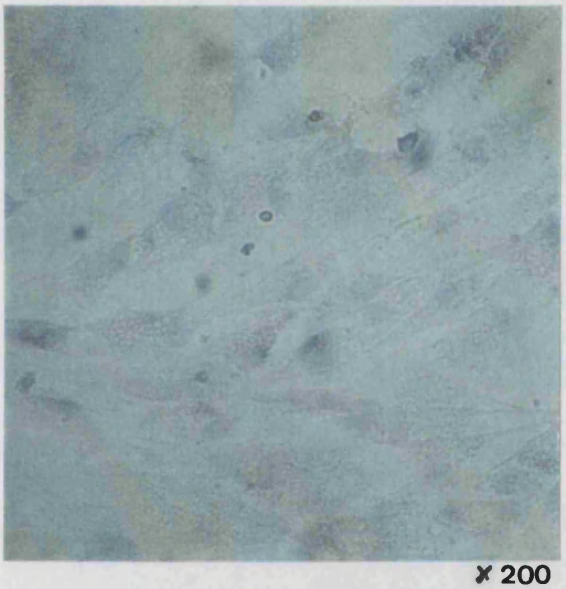
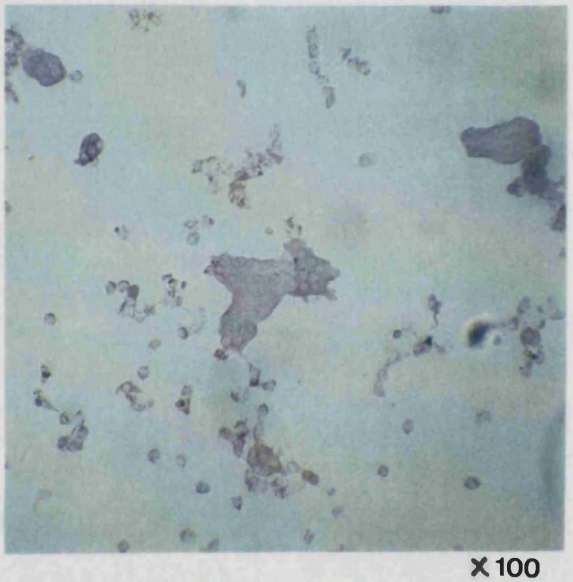
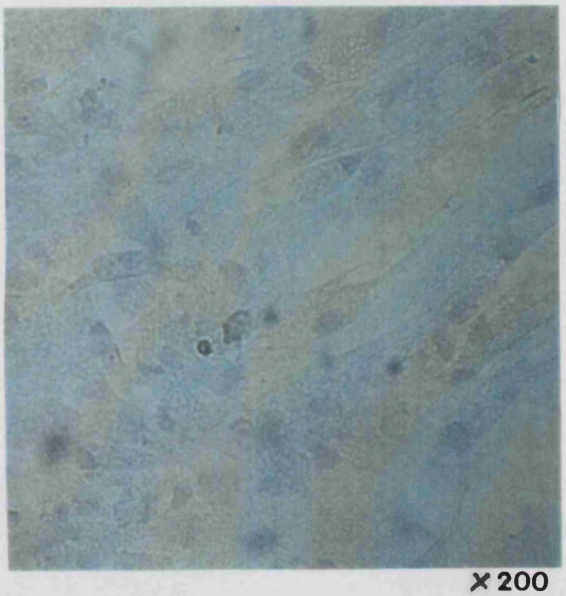

B
× 100


C
× 200


D
× 100


E
× 200

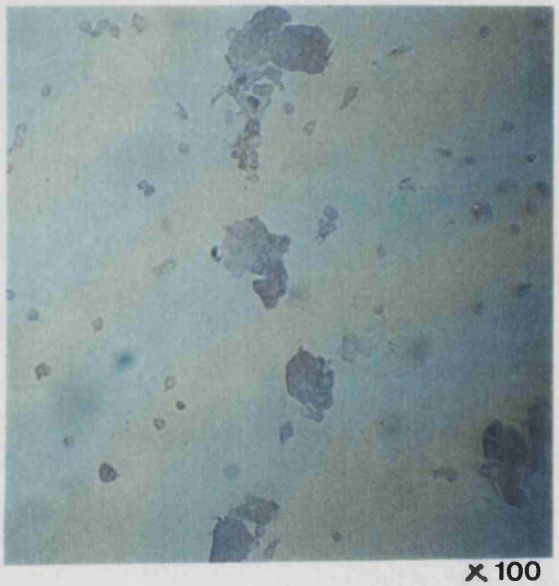

F
× 100

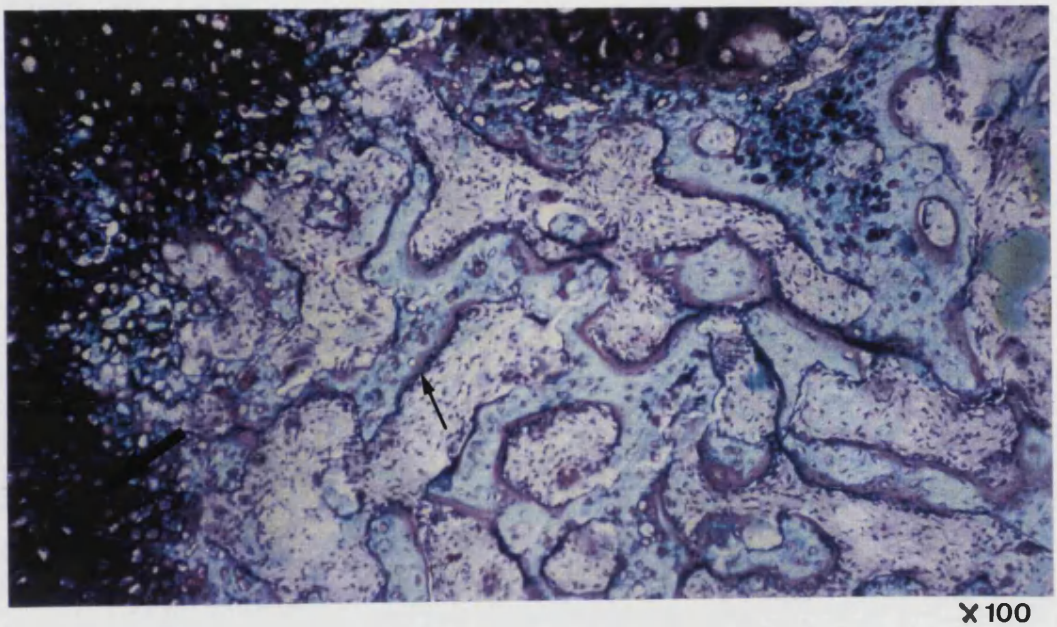
Fig. 5.5 IL-6 is present in osteoblasts and populations of chondrocytes in the osteophyte.

Fig. 5.5a Wrights stain of a section of endochondral bone formation in an osteophyte. Cartilage (large arrow) is eroded by osteoclasts and replaced by osteoblasts depositing woven bone. Woven bone is subsequently resorbed and replaced with lamellar bone closely lined by osteoblasts (small arrow).

Fig. 5.5b Anti-sense section of a similar area of bone showing plump osteoblasts expressing large amounts of IL-6 mRNA (small arrow) in comparison to more flattened osteoblasts apposed to the bone surface (large arrow). A population of chondrocytes also express IL-6 mRNA (double arrow).

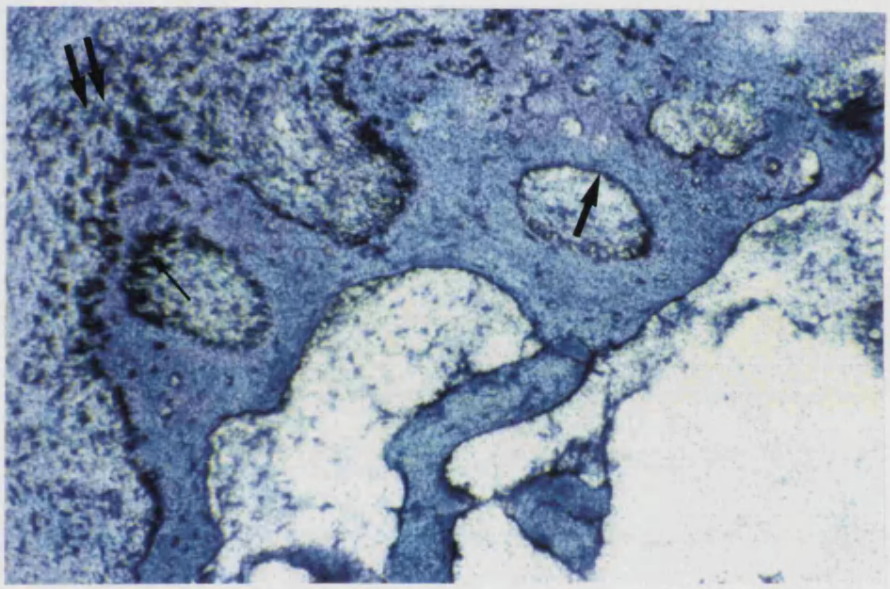
Fig. 5.5c Sense probe exhibited no binding.

a



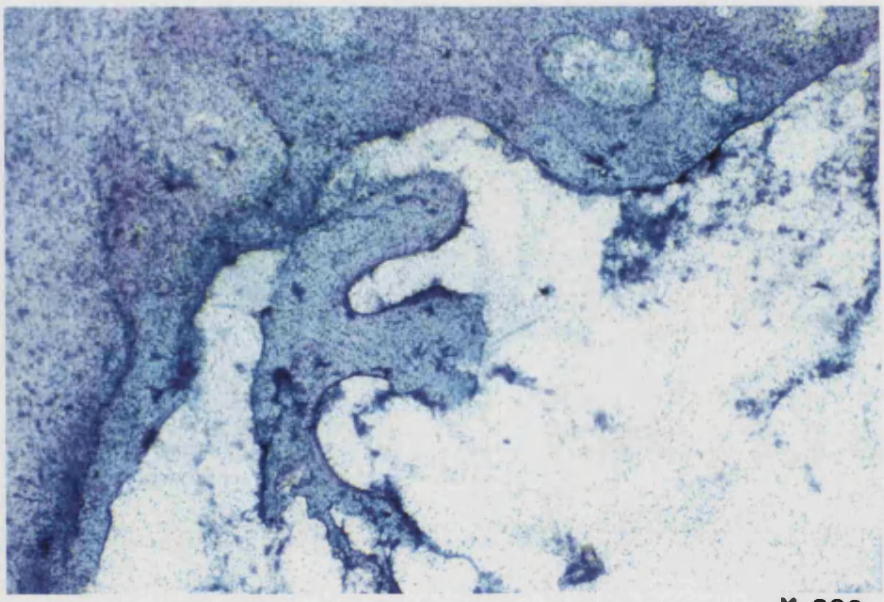
X 100

b



X 200

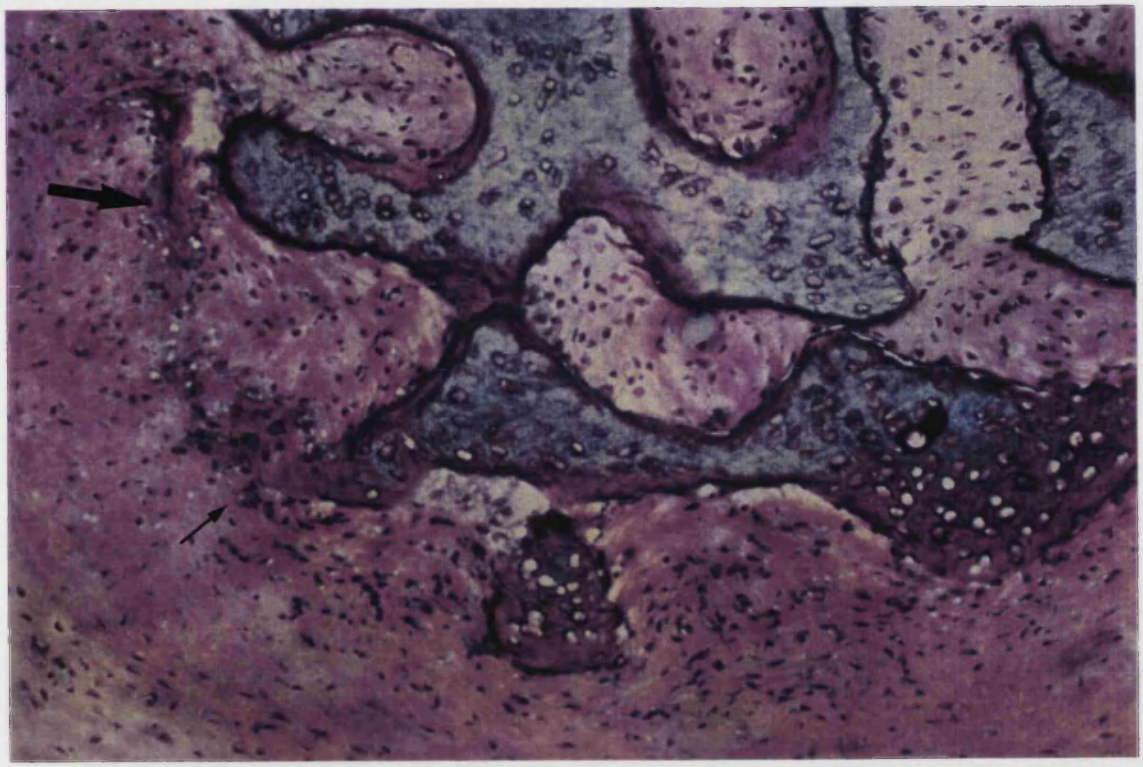
c



X 200

Fig. 5.6 IL-6 is present in newly formed osteoblasts in the osteophyte.

a



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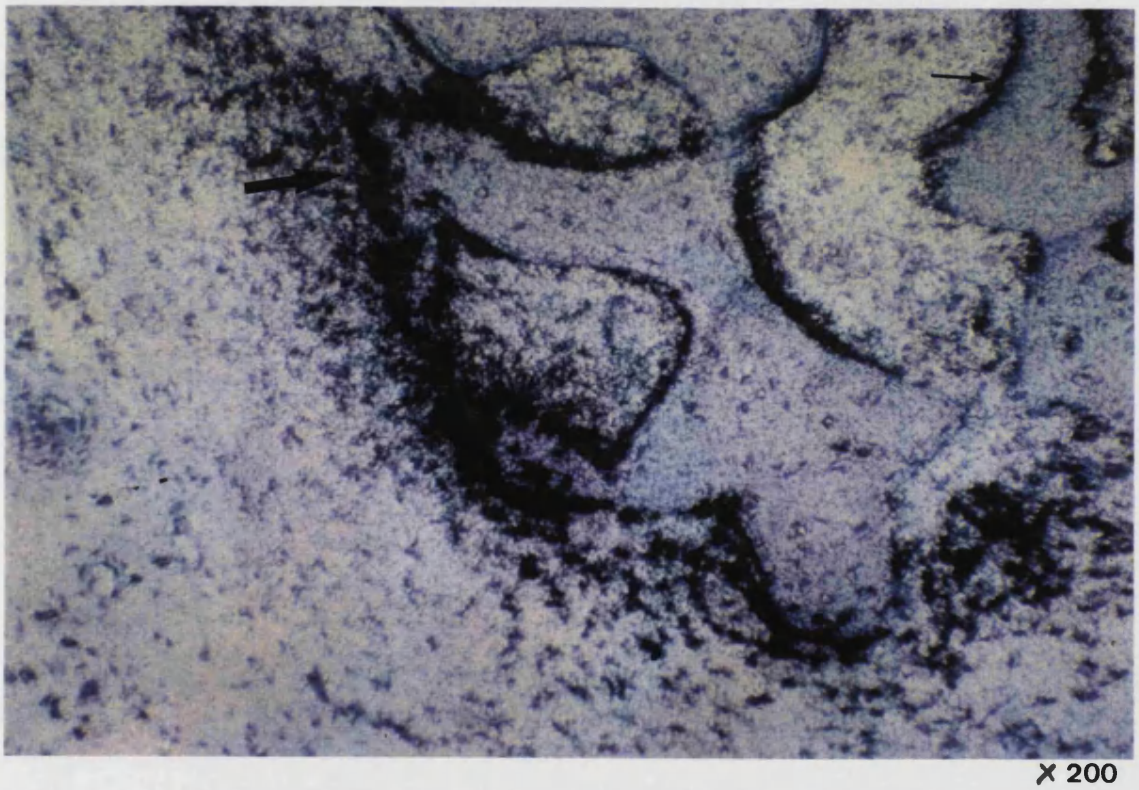


X 200

Fig. 5.6a Wrights stain of an area of intramembranous bone formation in the developing osteophyte. A fine layer of osteoblasts (large arrow) derived from the surrounding connective tissue (small arrow) are actively laying down woven bone.

Fig. 5.6b Anti-sense section of a similar area showing high expression of IL-6 mRNA in the newly formed plump osteoblasts (large arrow) compared to the more mature osteoblasts apposed to the bone surface (small arrow).

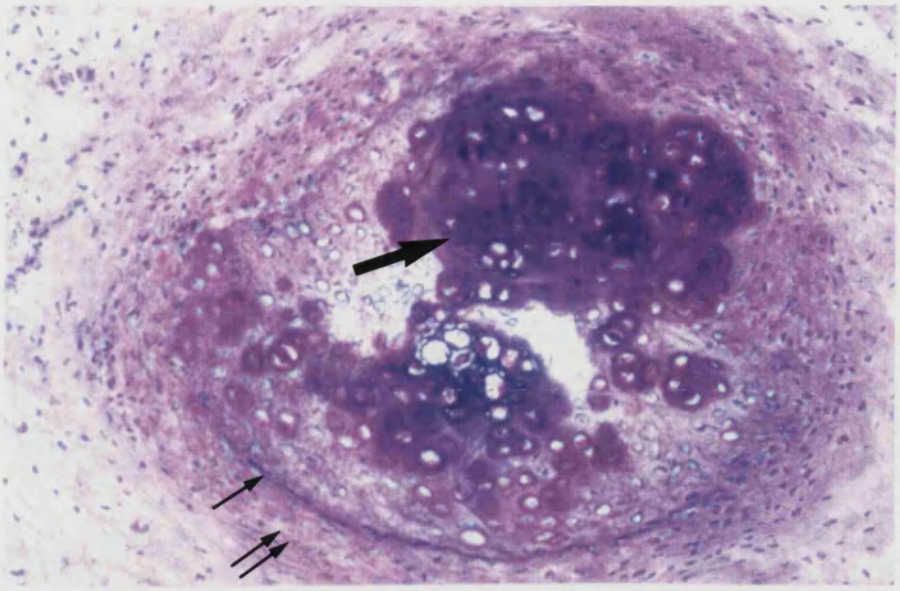
Fig. 5.7 IL-6 is present in newly formed osteoblasts in the tip of a developing osteophyte.

Fig. 5.7a Wrights stain of the tip of a developing osteophyte. Both endochondral and intramembranous bone formation processes are evident. Cartilage (large arrow) is eroded and replaced by woven and subsequently lamellar bone. A thin layer of osteoblasts (small arrow) have differentiated from the surrounding connective tissue (double arrow).

Fig. 5.7b Anti-sense section showing high expression of IL-6 mRNA in the newly formed osteoblasts (large arrow) derived from the connective tissue.

Fig. 5.7c Sense probe exhibited no binding.

a



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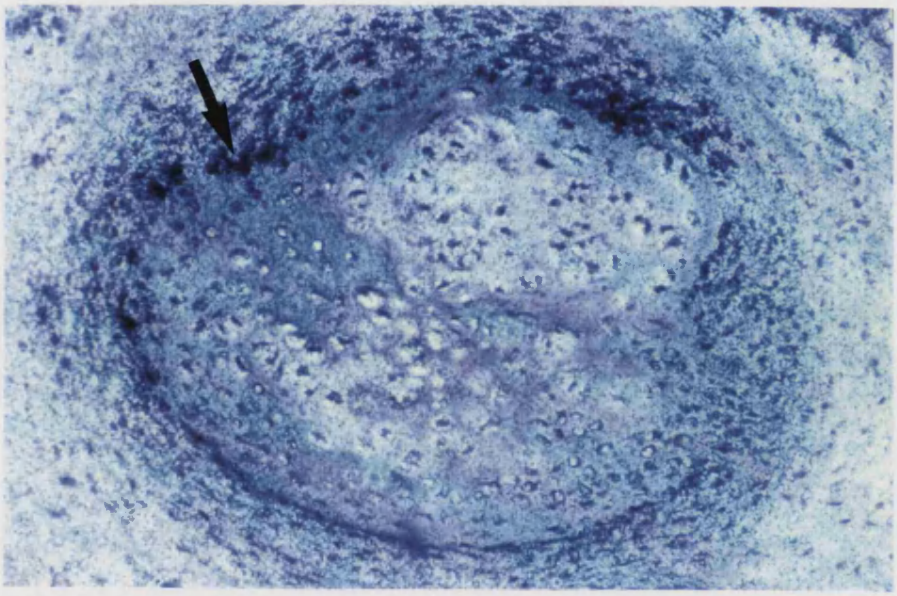

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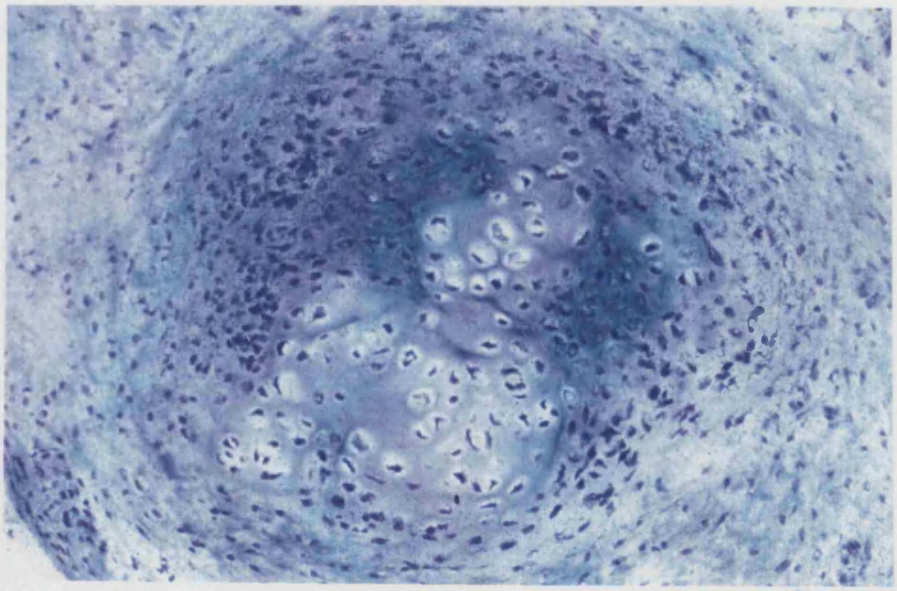


× 100

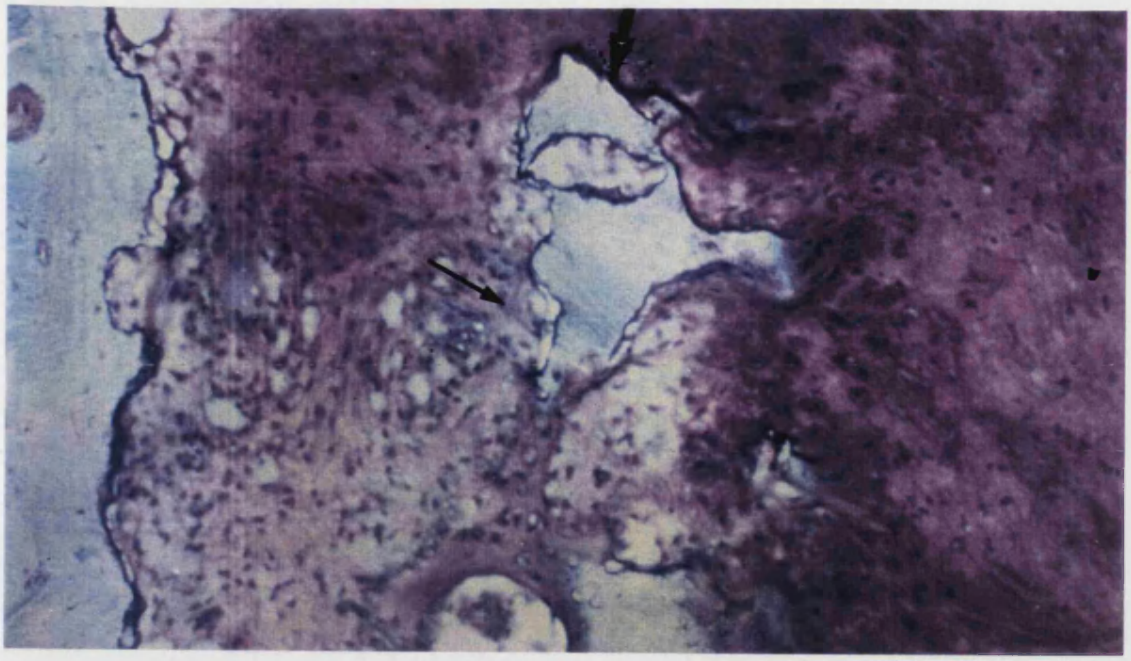
Fig. 5.8 IL-6 is present in cells within the maturing osteophyte.

Fig. 5.8a Histology of a section of osteophyte containing a spicule of newly formed bone lined by osteoblasts (large arrow) and osteoclasts (small arrow).

Fig. 5.8b IL-6 mRNA was present in osteoblasts, osteoclasts, marrow cells and a population of chondrocytes.

Fig. 5.8c Sense probe exhibited no binding.

a



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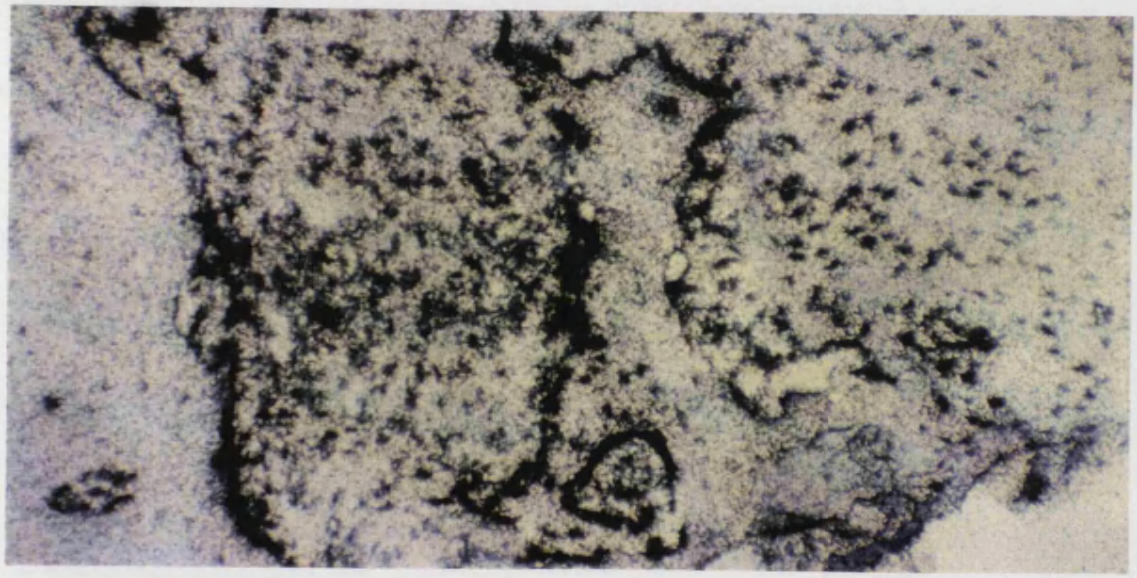

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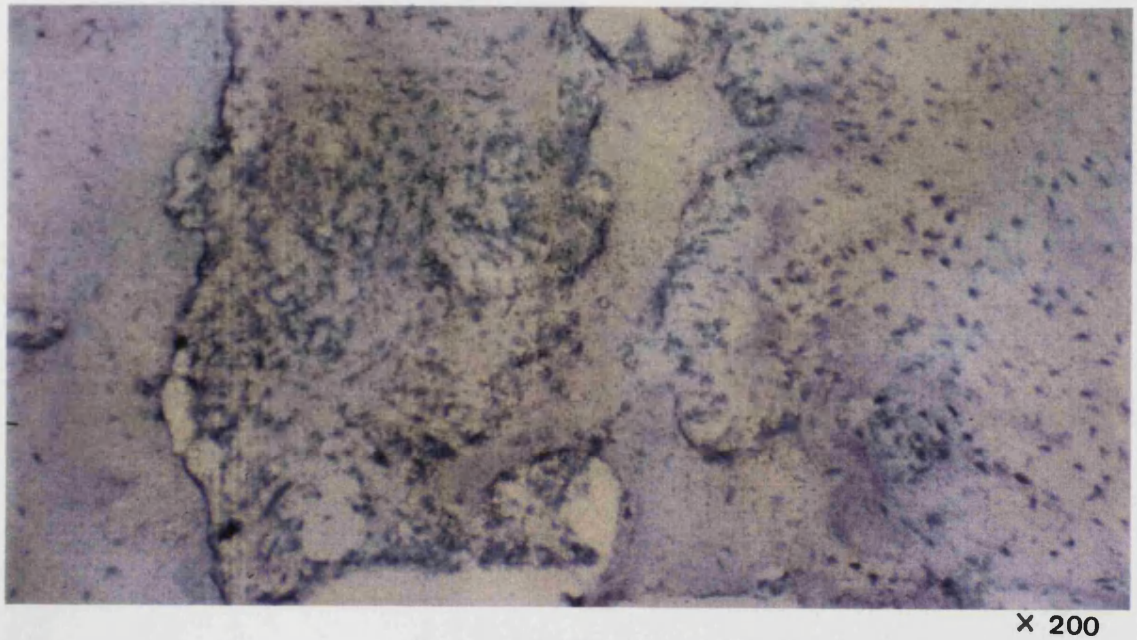


× 200

Fig. 5.9 IL-6 is present in osteoclasts within the osteophyte.

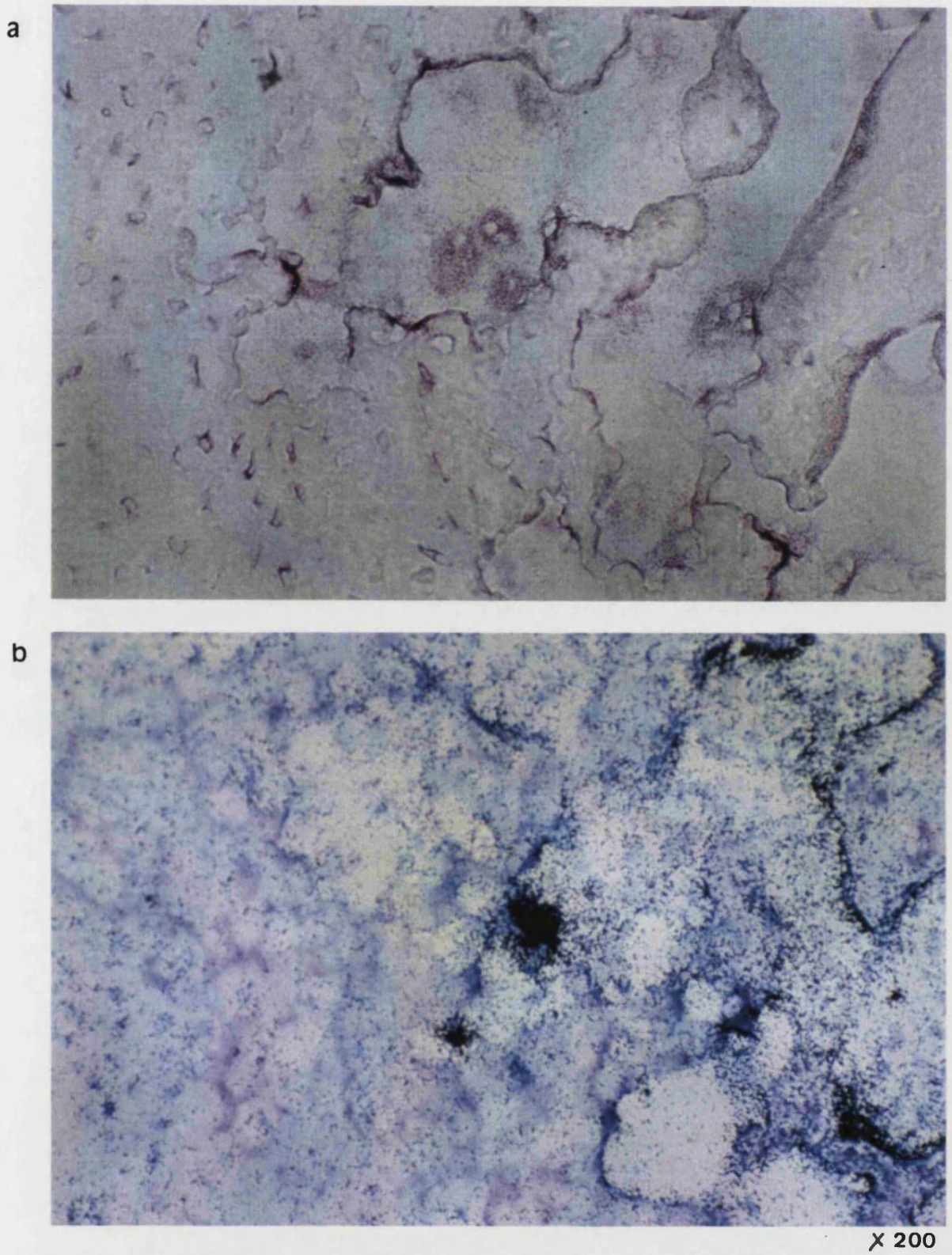
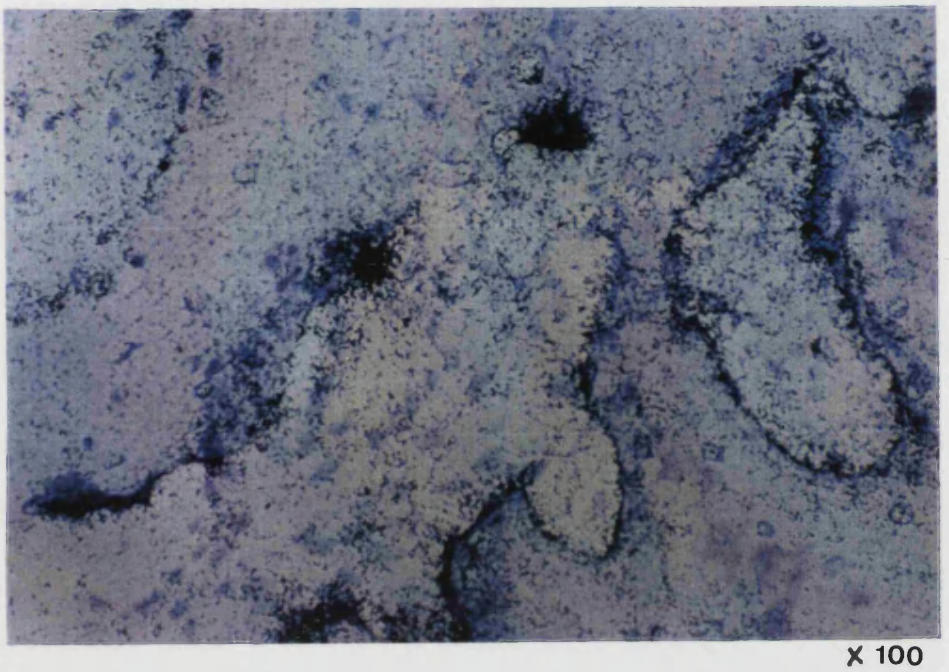


Fig. 5.9a Section of osteophyte reacted for TRAP activity demonstrates the presence of osteoclasts within an area of endochondral bone formation.

Fig. 5.9b Similar area of osteophyte showing high levels of IL-6 mRNA expression in osteoclasts.

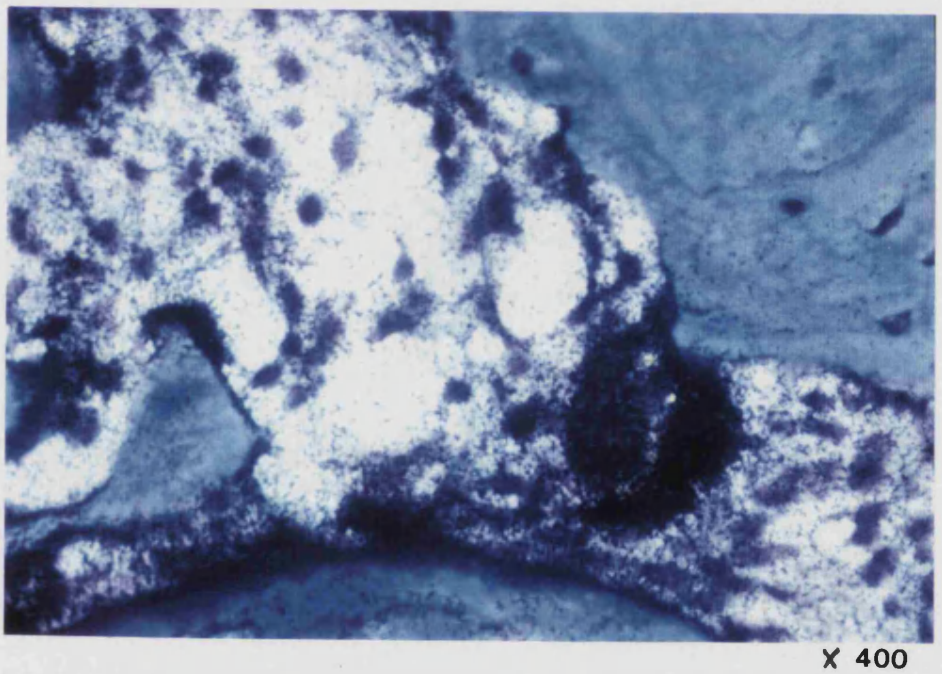
Fig. 5.10 IL-6 is present in osteoclasts within various osteophytes.

a



X 100

b



X 400

Fig. 5.10 Osteoclasts adjacent to the bone surface within different osteophytes express high levels of IL-6 mRNA expression.

Fig. 5.11 IL-6 is present in osteoclasts within osteoclastoma tumour.

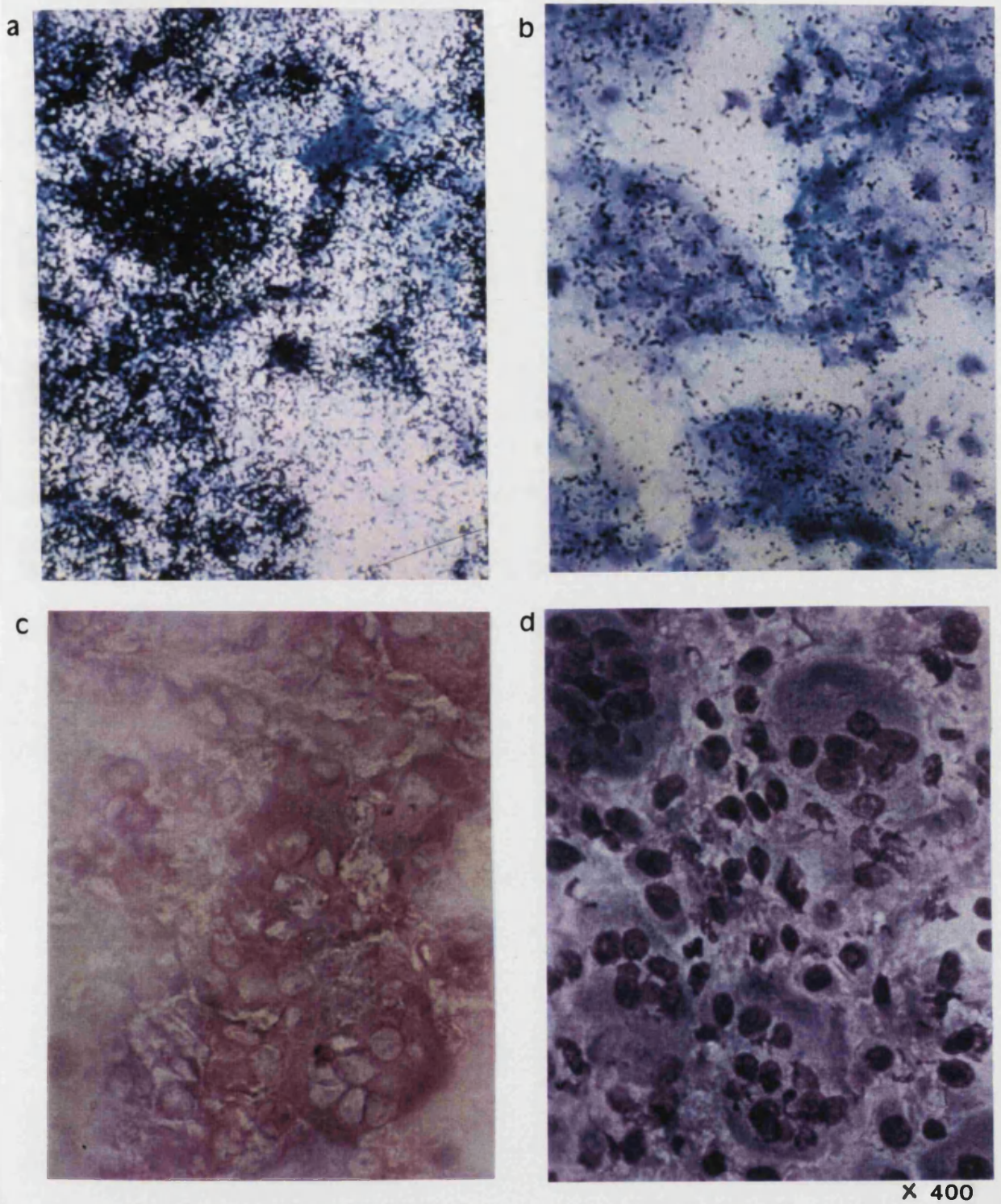


Fig. 5.11a Osteoclasts and stromal cells within an osteoclastoma express high levels of IL-6 mRNA.

Fig. 5.11b Sense probe exhibited no binding.

Fig. 5.11c A similar area of tumour was reacted for TRAP activity to confirm the presence of osteoclasts.

Fig. 5.11d Histology of the tumour also demonstrates the presence of the multinucleated osteoclasts.

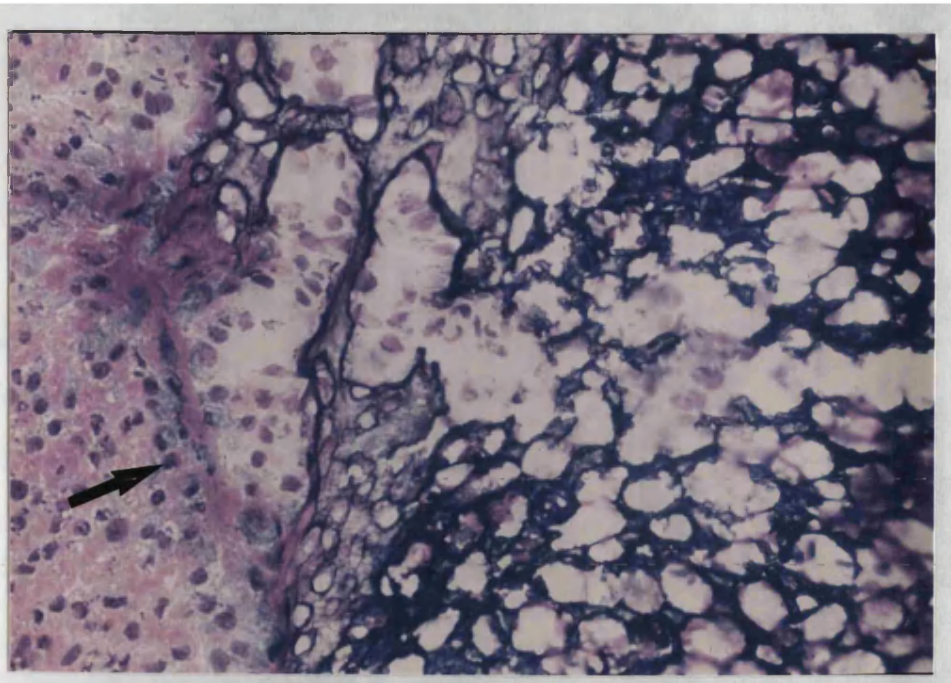
Fig. 5.12 IL-6 is present in osteoblasts within fetal tissue.

Fig. 5.12a Histology of fetal rib showing osteoblasts derived from the connective tissue during intramembranous bone formation (large arrow) laying down woven bone.

Fig. 5.12b IL-6 mRNA is present in high levels newly formed osteoblasts (large arrow).

Fig. 5.12c Sense transcript exhibited no binding.

a



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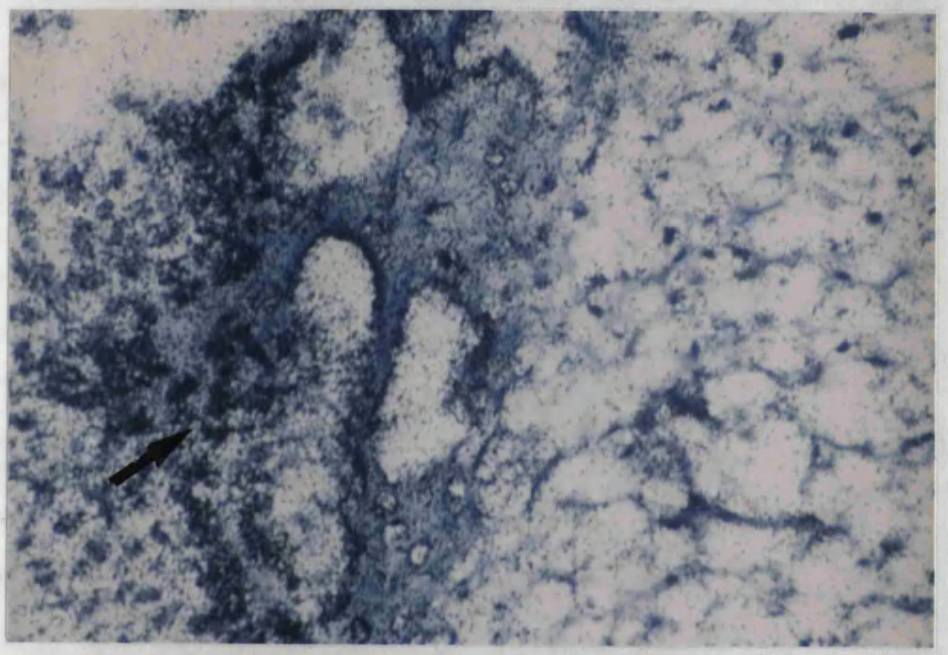
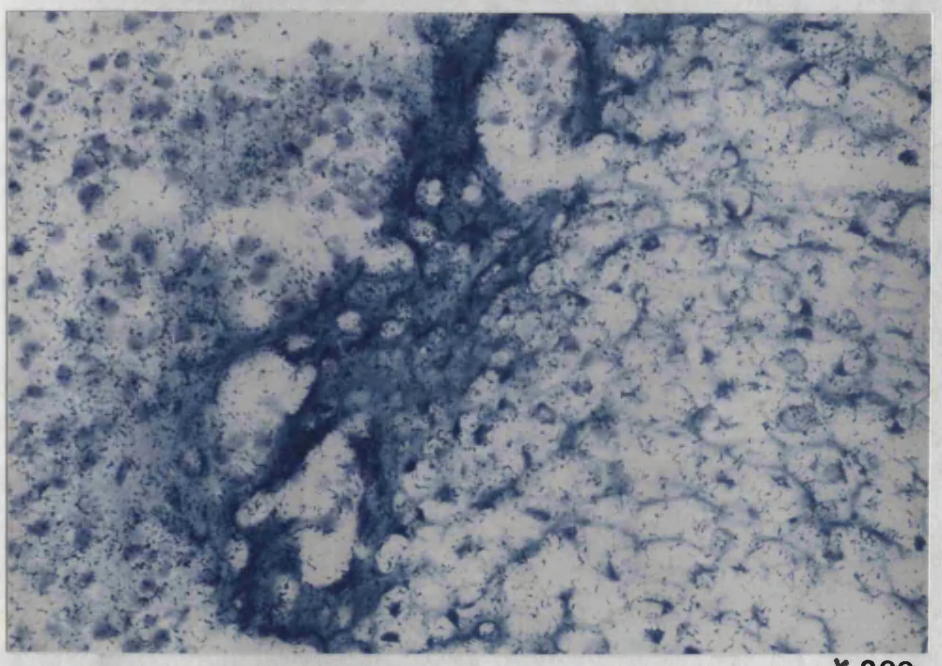


c



X 200

APPENDIX 4

Alkaline phosphatase substrate solution

Napthol AS-MX phosphate, free acid	2 mg
N,N-dimethylformamide	0.2 ml
0.1 M Tris buffer pH 8.2	9.8 ml
1 M Levamisole	0.04 ml
Fast Red TR salt	10 mg

This solution was made by dissolving Napthol in N,N-dimethylformamide in a glass tube. Tris buffer was added followed by the levamisole (blocked endogenous bone alkaline phosphatase, but not intestinal calf alkaline phosphatase present in streptavidin/alkaline phosphatase). The solution was adjusted to pH 8.2. Immediately before use, the fast red TR salt was dissolved in this solution and it was filtered onto the slides.

3-aminopropyltriethoxysilane (TESPA) coating of slides

Slides were handled with gloves and prepared by dipping into the following solutions:-

10% (v/v) HCl in 70% (v/v) alcohol for 10 seconds

Distilled water for 10 seconds

95% (v/v) alcohol for 10 seconds

The slides were dried in an oven at 150°C and allowed to cool before dipping into the following solutions:-

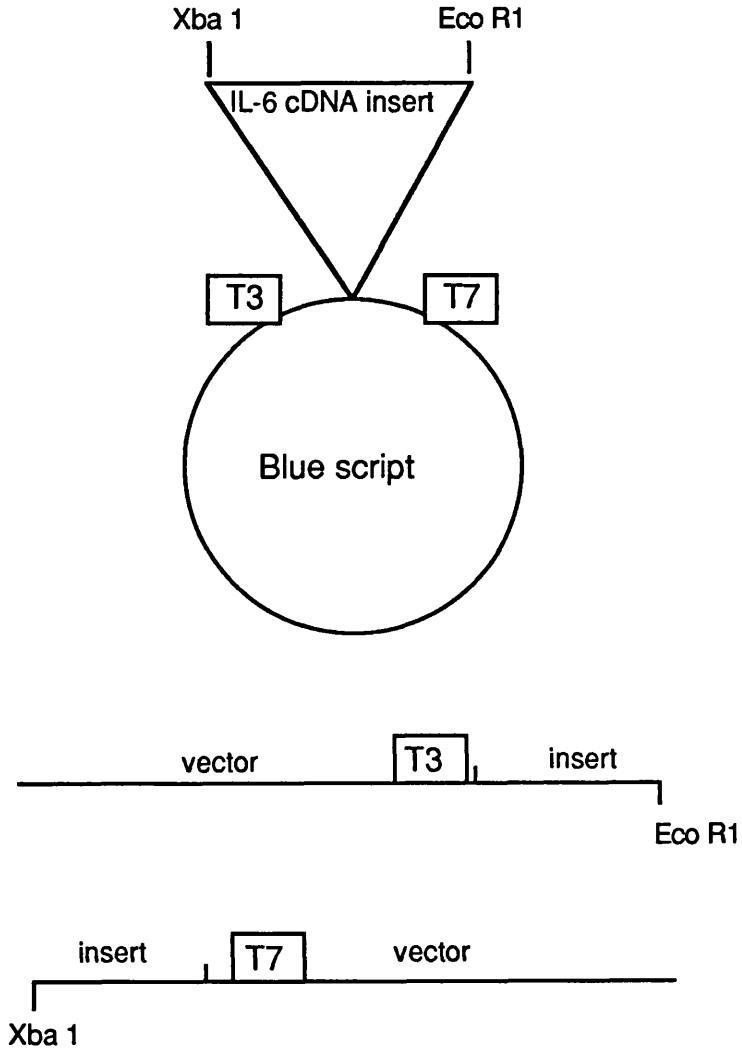
2% (v/v) TESPA in acetone for 10 seconds

100% acetone for 2 X 10 seconds

Distilled water for 10 seconds

The slides were baked dry in an oven at 42°C,transferred to a dessicated box and stored at 4°C.

Different linear species obtained by cutting the vector/insert intersection with two different enzymess



Hybridisation Buffer

50% formamide

2 XSSC

10% dextran sulphate

10 mM DTT

RNase inhibitor - 0.4 U/ml

10 U tRNA

1 X denharts solution (see Appendix 3)

200 ug/ml salmon sperm DNA (see Appendix 3)

1 X T4 DNA polymerase buffer

16.6 mM ammonium sulphate

37 mM Tris HCl pH 8.8

6.7 mM magnesium chloride

10 mM 2-mercaptoethanol

6.7 uM EDTA

33 uM ATP, CTP, GTP, TTP

170 ug/ml BSA

CHAPTER 6

EXPRESSION AND MODULATION OF IL-6R IN HUMAN OSTEOBLAST-LIKE CELLS.

ABSTRACT

As shown previously (Chapter 3), IL-6 does not modulate osteoblast function and one explanation for this may be related to the lack of IL-6 receptor (IL-6R) expression by these cells. The IL-6R has been recently cloned and cDNA probes are available for the study of the expression of the IL-6R. IL-6R has been shown to be expressed in many cell types including myeloma cells, hepatocytes and lymphocytes. The expression and modulation of IL-6R mRNA in human osteoblast-like cells has therefore been investigated. Total RNA was extracted from unstimulated human osteoblast-like cells and from cultures stimulated with rhIL-1α (10^{-11} M), rhTNFα (10^{-8} M), IL-6 (2000 pg/ml), 1,25$(OH)_2D_3$ (10^{-8}M) and hPTH (1-34) (10^{-9} M). Northern and dot blots were probed for IL-6R using a specific cDNA probe. The probe annealed to a hybridising species which was heavier than the 28S rRNA band and was composed therefore of approximately 5000 - 6000 nucleotides confirming its identity (Yamasaki et al 1988, M. Baumann et al 1990). IL-6R mRNA was constitutively produced by the human osteoblast-like cells and none of the osteotropic agents tested modulated its expression over a 24 h incubation period in either serum free or 10% (v/v) FCS-containing EMEM. Dexamethasone (10^{-6} M) exerted no modulatory effect on the expression of IL-6R mRNA either in the presence or absence of rhIL-1α (10^{-11} M) or rhTNFα (10^{-8} M), unlike its effect on IL-6 mRNA production. These studies indicate that cultures of human osteoblast-like cells express IL-6R mRNA. The reason for the potential presence of receptors on osteoblast-like cells is unclear as no function for this cytokine has been found in these cells. Whether the mRNA for

the IL-6R is subsequently translated into a functionally responsive IL-6R protein is not currently known. It is possible that associated components required for IL-6/IL-6R interactions may be absent or that processes involved in signal transduction may be defective. Alternatively, excess IL-6 levels produced by these cells (Chapter 4) may exert a desensitising effect on the receptor. Further studies are required to address these individual possibilities.

INTRODUCTION

The receptor for IL-6 was first cloned from human leukocytes (Yamasaki et al 1988) and from the nucleotide sequence, its amino acid structure has been determined. IL-6R consists of a 468 amino acid sequence including a hydrophobic signal peptide of approximately 19 amino acids and a poly A sequence usually associated with mRNA stability on the 3' end. Another hydrophobic area was located and this was presumed to be the transmembrane domain which was followed by an anchor region (Yamasaki et al 1988). The rat liver IL-6 receptor was recently cloned (Baumann et al 1990b). This group observed that the rat IL-6R possessed areas of homology with the human counterpart such as the signal peptide region and the transmembrane domain. IL-6R also shares several structural features with a cytokine receptor family which included GM-CSF, murine IL-4 (mIL-4), mIL-3 and hIL-2 (B chain) (Baumann et al 1990b).

The detection of receptors for IL-6 first was reported by Taga et al (1987). Using radio-iodinated IL-6, they demonstrated the existence of between 200 - 2700 IL-6R sites on a human B cell line SKW6-CL4, Epstein Barr virus transformed B-cell lines, U937 and HL-60 cells. They also located IL-6R on resting T cells although the number of sites per cell decreased upon activation. This correlates well with evidence for early effects of IL-6 on T cell proliferation (Ceuppens et al 1988, Tosato and Pike 1988; refer to introduction for further references). Evidence for

the late activation of T cells by IL-6 (Okada et al 1988) may be due to different responding subsets of T cells (Garman et al 1987, Hodgkin et al 1988, Habetswallner et al 1988).

IL-6R are not present on resting human B cells in the absence of stimulation although receptor number increases upon activation of B cells. This fits in well with the late acting function of IL-6 on the terminal differentiation of B cells into immunoglobulin producing cells (Takatsuki et al 1988, Muraguchi et al 1988, Kishimoto and Hirano 1988 for review).

IL-6R have also been located on a number of other cells including hepatocytes (Rose-John et al 1991, M. Baumann et al 1990, Snyers et al 1990, Bauer et al 1989), rat and human skin fibroblasts (Rose-John et al 1991), myeloma cells (Taga et al 1987, Yamasaki et al 1988, Taga et al 1989, Kawano et al 1988) and human blood mononuclear cells (Bauer et al 1989). Therefore, like IL-6, the receptor is expressed by a number of different cell types. Several groups have demonstrated a glucocorticoid up-regulation of IL-6R in hepatoma cell lines which is in accordance with an increased acute phase protein production by glucocorticoids (Snyers et al 1990, Bauer et al 1989). In contrast Bauer et al (1989) described a down regulation of IL-6R by glucocorticoids in human blood monocytes. Bauer et al (1989) also observed that IL-6 itself is able to reduce IL-6R mRNA in human monocytes, and increase it in human hepatocytes.

IL-6 is also known to be an autocrine growth factor for some myelomas (Klein et al 1987, Kawano et al 1988, Sidell et al 1991). This would explain the presence of IL-6 receptors and production of this cytokine in myeloma.

The observations that no detectable responses to IL-6 can be demonstrated in human osteoblast-like cells (Chapter 3) may indicate the absence of receptors for this cytokine. In this chapter, the expression of IL-6R mRNA was examined in

human trabecular bone cells in response to various osteotropic agents including dexamethasone and IL-6.

METHODS

RNA extraction, Northern and dot blot analysis were conducted as described in Chapter 4 methods.

The concentration for each osteotropic agent used was based on IL-6 bioassay data with the exception of dexamethasone and IL-6. In the case of IL-6, the concentration was based on endogenous levels produced by human osteoblast-like cells under serum-free conditions measured by bioassay (Chapter 4). The concentration of dexamethasone used was obtained from recent work demonstrating a dexamethasone down regulation of IL-6 bioactivity in fibroblasts (Waage et al 1990).

RESULTS

The IL-6R probe was verified by Northern blot analysis using unstimulated human osteoblast-like cells. The hybridising species was heavier than the 28S rRNA band and corresponded to approximately 5 - 6 kb (fig. 6.1). The receptor for IL-6 is a much larger protein than its ligand (80 kDa vs 26 kDa) respectively and this was reflected in the different sizes of the mRNA species (approx. 5 kb compared to 1.3 kb) (fig. 6.1).

IL-6R mRNA was constitutively expressed in very high levels compared to IL-6 mRNA in human osteoblast-like cells (fig. 6.1). It was not modulated by rhTNFα (10^{-8} M) in serum-free containing medium over a 24 h time course (fig. 6.2). Furthermore, this cytokine did not regulate IL-6R mRNA levels in EMEM containing 10% (v/v) FCS although the time couse was only studied for the first 8 h (fig. 6.3). The effects of rhIL-1α (10^{-11} M) over 48 h were studied in EMEM

containing 10% (v/v) FCS. IL-6R mRNA remained at constitutive background levels throughout the whole culture period (fig. 6.4). When excess exogenous IL-6 (2000 pg/ml) was added to human osteoblast-like cells in serum-free culture, no receptor modulation was observed over the 24 h incubation period (fig. 6.5). $1,25(OH)_2D_3$ at a concentration of 10^{-8} M exerted no effect on the IL-6R mRNA levels over 24 h serum-free culture period (fig. 6.6). The paler dot after 2 h treatment was due to a loading error - the first 3 rows show clearly a lack of modulation by this hormone. hPTH (1-34) at a concentration of 10^{-9} M did not alter the constitutive expression of IL-6R mRNA over 24 h serum-free culture period (fig. 6.7) and in EMEM containing 10% (v/v) FCS (fig. 6.8). The effect of dexamethasone (10^{-6} M) alone and in combination with rhIL-1α (fig. 6.9) and rhTNFα (fig. 6.10) on IL-6R mRNA levels was investigated. Under serum-free conditions the glucocorticoid exerted no regulatory effect on the basal IL-6R mRNA levels in the presence or absence of rhIL-1α or rhTNFα.

DISCUSSION

These studies indicate that although IL-6R mRNA was present constitutively, none of the agents tested modulated the mRNA in human osteoblast-like cells. Glucocorticoids and IL-6 neither stimulated nor inhibited IL-6R mRNA levels in osteoblasts which is in contrast to their stimulatory effects on other cells such as hepatocytes (Bauer et al 1989, Snyers et al 1990). Bauer et al (1989) also observed a down regulation of IL-6R in human blood monocytes by IL-6, IL-1 and dexamethasone and an upregulation of IL-6R mRNA in hepatocytes by these agents. From these studies they concluded that monocytes bind trace IL-6 circulating in the serum, but during an inflammatory response, IL-6 binds preferentially to hepatocytes and so it therefore follows that the number of IL-6R sites/cell will be up regulated. Circulating glucocorticoids have a protective role

which is mediated by increasing acute phase protein production and IL-6R number which in turn will increase the hepatocyte response to IL-6. Sanceau et al (1991) reported that in human monocytic THP-1 cells, IFNγ increases IL-6R mRNA but IFNγ and TNFα decrease the levels of IL-6R mRNA. Since levels of IFNγ and TNF will be increased during inflammation, the work of Sanceau et al (1991) supports that of Bauer et al (1989) in that the target cell for IL-6 may be shifted from monocytes to other cells such as hepatocytes. The fact that B-cells acquire IL-6R upon activation, relates to the immunomodulatory function of IL-6 as a B cell maturation factor (Takatsuki et al 1988, Muraguchi et al 1988). Therefore in the above studies, the up regulation of IL-6R appears to correlate with an immunological role of IL-6.

In contrast, no function for IL-6 was determined in human osteoblast-like cells (chapter 3). This may have been due to the fact that inappropriate parameters were studied. Another possibility is evident from the studies presented in this chapter which only demonstrate the presence of IL-6R mRNA and not the mature IL-6R protein. The IL-6R mRNA may not be translated and expressed as the functional membrane receptor. Many other groups have observed the presence of various different mRNA species in a number of cells, but have been unable to detect the final protein (Garrett et al 1987, McDonald and Tuan 1989, Hakeda et al 1991). In order to demonstrate whether the human osteoblast-like cells express IL-6R protein, attempts are in progress to investigate the presence of the IL-6R protein in these cells by the use of immunoprecipitation techniques.

An alternative theory is that the receptor is translated into the final mature protein, but the signal transduction mechanism is absent. Interestingly, the IL-6R possesses no tyrosine kinase domain (Yamasaki et al 1988), nor does the ligand receptor complex appear to signal through the recognised channels such as increased intracellular Ca^{2+} and phosphoinositide turnover (M. Baumann et al

1990). Therefore a novel signal transduction mechanism may exist. Evidence for this is substantiated by Taga et al (1989) using a human myeloma cell line. Taga et al (1989) revealed by immunoprecipitation techniques using a specific anti-IL-6R antibody, that a 130 kDa glycoprotein, gp130 was capable of associating with the receptor after addition of IL-6. Furthermore, this association was necessary for signal transduction. This proposal was further supported by evidence from Baumann et al (1990b) who used the rat IL-6R gene and an acute phase protein gene fused to a chloramphenicol acetyltransferase (CAT) reporter construct. These were co-transfected into a number of lines including human hepatoma cells, monkey COS cells and mouse L cells. Baumann et al (1990b) observed that CAT activity was only increased, upon addition of IL-6, in the human hepatoma lines (Hep3B-2 and HepG2). This demonstrates that more than receptor ligand binding is required to establish a functional IL-6 signal cascade. A likely candidate for this signalling component is gp130. Taga et al (1989) observed that gp 130 was present in all IL-6 responsive cells. Furthermore, Taga et al (1989) using a truncated cDNA, expressed a soluble form of the 80 kD IL-6 receptor which was capable of binding IL-6 and transducing a functional signal in cells which only contained the gp 130 and not the IL-6R. It is possible that human osteoblast-like cells, even though they may possess the 80 kD IL-6R, lack the signalling transduction mechanism involving gp130. Hibi et al (1990) cloned the 130 kD protein and confirmed Taga's findings that the peptide was present in IL-6 responsive cells. There is increasing information about the gp 130 peptide and the nuclear factors induced by IL-6. How the two are linked to transduce the IL-6 signal, remains to be discovered although recently, there is a suggestion that a tyrosine phosphorylation of a 160 kD protein and a serine kinase are involved in intermediate steps leading to activation of the relevant nuclear factors (Kishimoto - personal communication). Furthermore, a 61 amino acid region in the cytoplasmic

section of the gp 130 was reported to be sufficient to transduce the IL-6 signal (Murakami et al 1991). In addition, by truncating the gp 130, a loss of signal transducing ability was demonstrated to correspond to a lack of tyrosine phopshorylation of gp 130 induced by IL-6 (Murakami et al 1991).

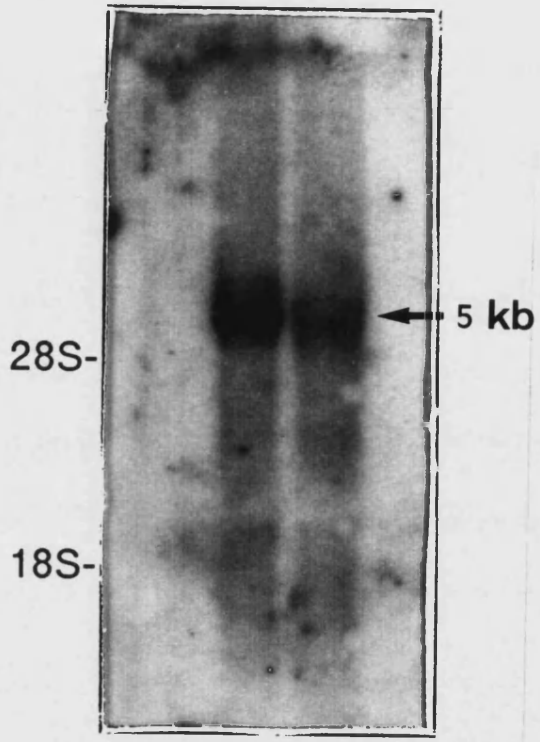
Gearing et al (1991) reported that although IL-6 and LIF were structurally distinct, there was a degree of homology between the gp 130 and the LIF receptor, with the transmembrane and cytoplasmic domains being most closely related. Gearing and co workers (1991) suggested that IL-6 and LIF may share common signal transduction pathways which would help to explain some of the similar activities of the two factors such as induction of differentiation of M1 cells (Miyaura et al 1989a, Metcalf 1989), stimulation of early hematopoiesis (Murray et al 1990) and induction of acute phase proteins from hepatocytes (Baumann et al 1989). Lord et al (1991) extended the studies of Gearing et al and investigated the intracellular signalling mechanisms induced by IL-6 and LIF during the differentiation of the myeloblastic leukemia M1 cells. Lord and co workers (1991) demonstrated that LIF and IL-6 shared a common intracellular signalling pathway which was distinct from other known mechanisms and involved a tyrosine phosphorylation which lead to activation of genes involved in myeloid differentiation.

There are differences between IL-6 and LIF actions on other cells however (see Chapter 1) and so the signalling pathways must vary in some respects. Possibly these differences may be related to the induction of alternative nuclear factors in different cells. The pleiotropic actions of IL-6 on different cell types may therefore be due to the expression of IL-6-inducible nuclear proteins (Baumann et al 1990b). IL-6 induces nuclear proteins IL-6DBP (IL-6 DNA binding protein) and NF-IL-6 (nuclear factor IL-6) in IL-6 responsive cells such as hepatocytes (Poli and Cortese 1989, Akira et al 1990b). In addition, these binding proteins are homologous, but distinct (Poli et al 1990). Therefore, some of the many variable actions of IL-6 in

different cell types may be due to the range of nuclear factors induced by this cytokine, as well as due to the presence of appropriate 'IL-6 responsive elements' in the 5' upstream region of IL-6 responsive genes.

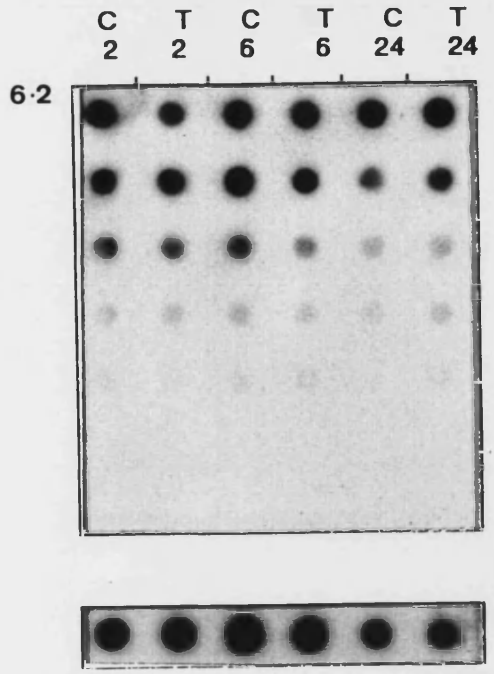
Since IL-6R mRNA levels were not modulated in human osteoblast-like cells by any of the agents within this chapter, one, or a combination of these above points are possible. Further work is required to address these points.

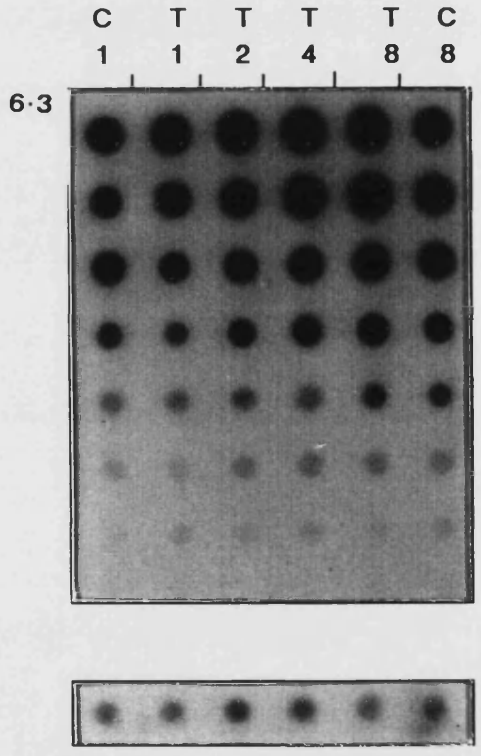
Fig.6.1 Constitutive expression of IL-6R mRNA by Northern blot in human osteoblast-like cells.



10ug of RNA from human trabecular bone cells was loaded for each of the 2 lanes. The resultant filter was probed for IL-6R (see Chapter 4 methods). The transcript was larger than the 28S ribosomal RNA band and was therefore approx. 5000 bases. This is a representative example of the twelve patients studied.

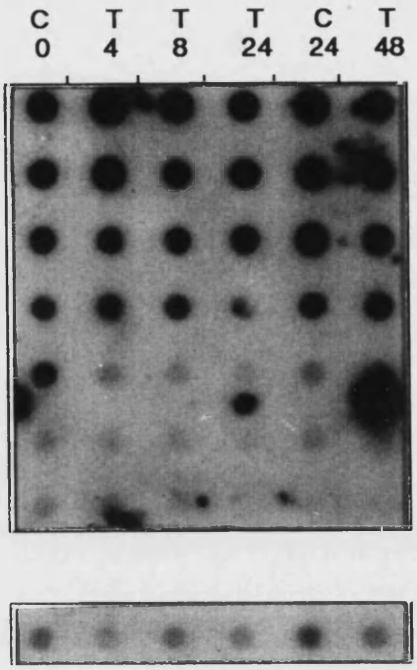
Lack of modulation of IL-6R mRNA by rhTNF∝in human trabecular bone cells in serum-free (fig. 6.2) or 10% FCS (fig. 6.3) containing EMEM.





rhTNF∝ (10^{-7}M) was incubated with human osteoblast-like cells for various time points and the total RNA was subsequently extracted. Serial two-fold dilutions of RNA were performed starting with 4ug of RNA as the highest concentration. After probing for IL-6R, the filter was stripped and reprobed with the cDNA probe for β actin. C = unstimulated osteoblast RNA and T = rhTNF∝.

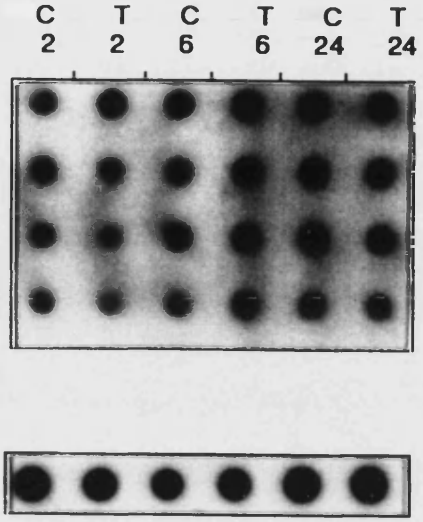
Fig 6.4 **Lack of modulation of IL-6R mRNA by rhIL-1α in human trabecular bone cells in 10% FCS containing EMEM.**



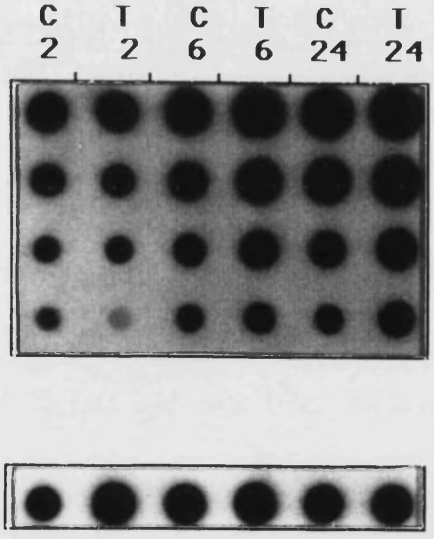
Human osteoblast-like cells were treated with rhIL-1α (10^{-11}M) for 4, 8, 24 and 48 h and total RNA was extracted. Serial two-fold dilutions of RNA were performed starting with 5ug of RNA. After probing for IL-6R mRNA, the filter was stripped and reprobed with the β actin cDNA probe. C = unstimulated osteoblast RNA and T = rhIL-1α treatment.

Lack of modulation of IL-6R mRNA by rhIL-6 (fig. 6.5) and $1,25(OH)_2D_3$ (fig. 6.6) in human trabecular bone cells in serum-free containing EMEM.

6.5

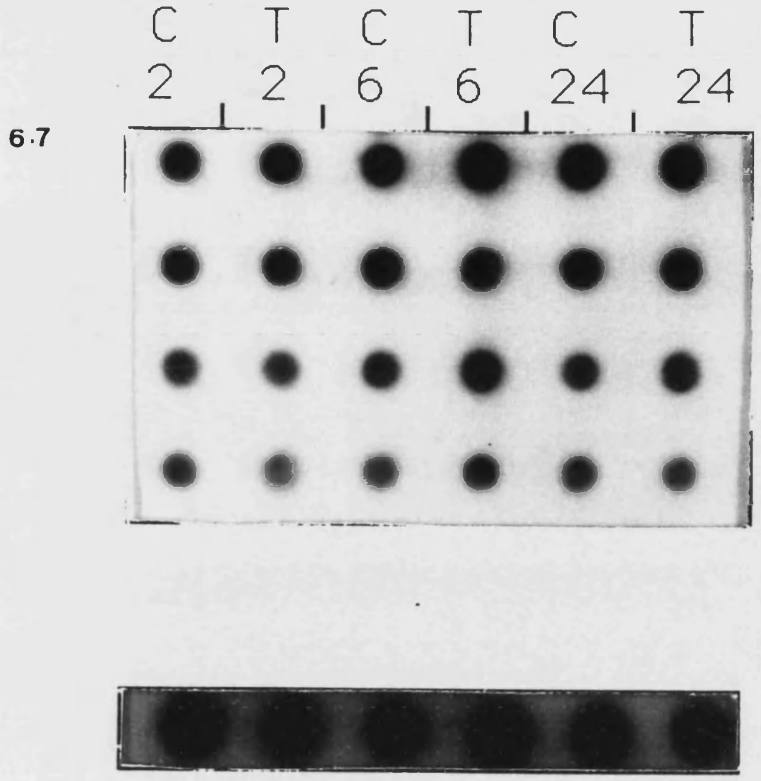


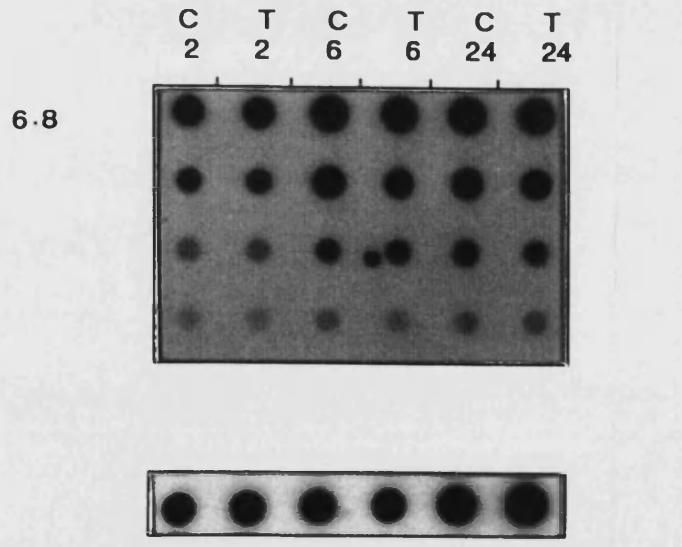
6.6



Human osteoblast-like cells were treated with $1,25(OH)_2D_3$ (10^{-8}M) or IL-6 (2000 pg/ml) for 2, 6 and 24 h and total RNA was extracted. Doubling dilutions starting at 6 ug of RNA were used and the filter probed for IL-6R and β actin mRNA.
C = unstimulated cells (vehicle) and T = treatment.

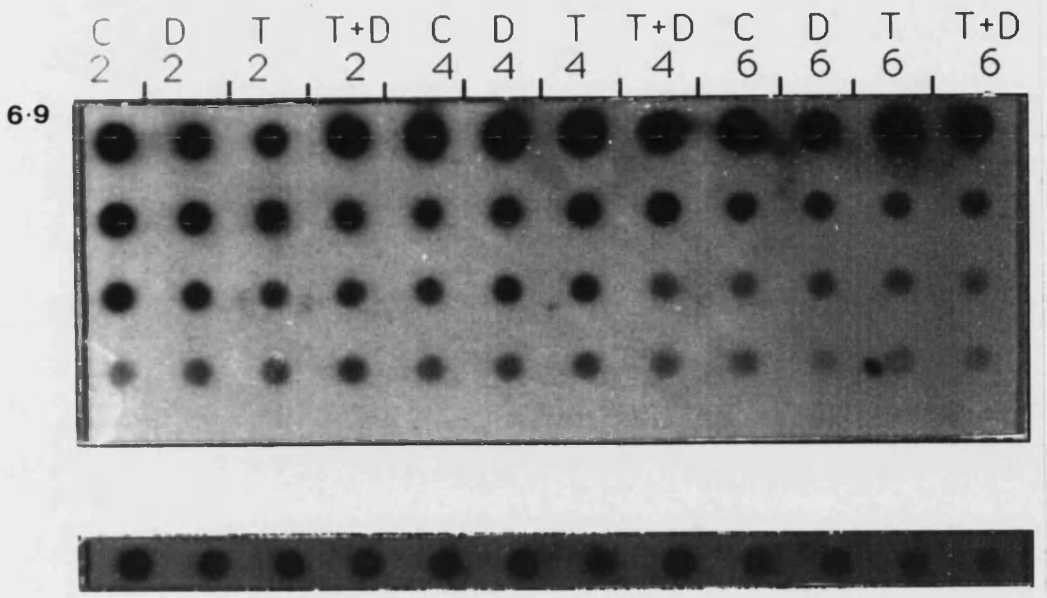
Lack of modulation of IL-6R mRNA by PTH in human trabecular bone cells in serum-free (fig. 6.7) and 10% FCS (fig. 6.8) containing EMEM.

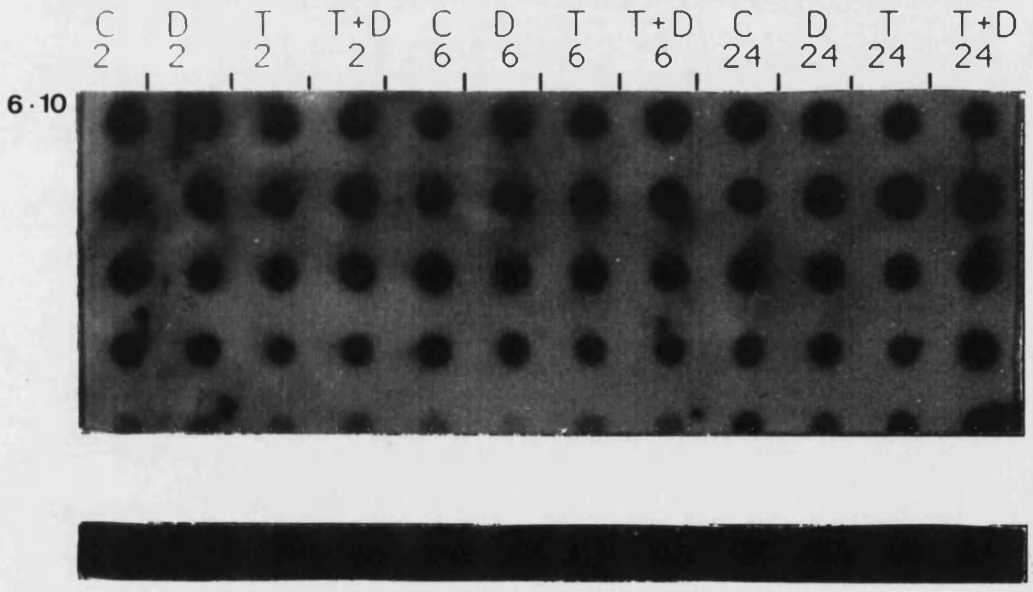




Human osteoblast-like cells were treated with PTH (10^{-9}M) for 2, 6 and 24 h and total RNA was extracted. Doubling dilutions starting with 3 ug of RNA were used. After probing with IL-6, the filter was stripped and reprobed with β actin. C = unstimulated cells (vehicle) and T = treatment.

Effect of rhIL-1∝(fig. 6.9) and rhTNF∝ (fig. 6.10) alone and in combination with dexamethasone on IL-6R mRNA and protein levels in human trabecular bone cells under serum-free conditions.





The osteoblasts were treated with rhIL-1∝(10^{-11}M), rhTNF∝(10^{-7}M) alone and in combination with dexamethasone (10^{-6}M) for various time points and the total RNA was extracted. Doubling dilutions starting with 4ug of RNA were used.
The filter was probed for IL-6R mRNA followed by βactin. C = unstimulated cells (vehicles), D = dexamethasone and T = treatment (rhIL-1∝and rhTNF∝).

CHAPTER 7

THE ROLE OF ENDOGENOUS IL-6 ON HUMAN OSTEOBLAST-LIKE CELL ACTIVITY.

ABSTRACT

In the work presented in Chapter 3, no potential function of exogenous IL-6 could be demonstrated on the activity of human and rat osteoblast-like cells. Furthermore, IL-6 did not modulate the activity of any stimulated osteoblast parameters measured. However, these cells also produce high basal endogenous levels of this cytokine as demonstrated in chapters 4 and 5. This raises the possibility that the high endogenous production of IL-6 may have masked the potential action of exogenous IL-6 on these cells. Endogenous IL-6 synthesis may also mediate the effects of osteotropic agents such as IL-1α and TNFα on osteoblast-like cells. To investigate this possibility, a sheep anti-human IL-6 neutralizing antibody was used to clarify the role of endogenous IL-6 on various osteoblastic parameters. When added to human osteoblast-like cell cultures at a 1:1000 dilution, complete neutralization of endogenous IL-6 bioactivity, as assessed by the B9 assay, was observed. Various osteoblastic parameters were then studied in the presence and absence of this antibody. The contribution of basal endogenous IL-6 synthesis to the induction of osteocalcin production and alkaline phosphatase activity in response to $1,25(OH)_2D_3$ was examined. In addition, the involvement of the induction of IL-6 synthesis on the stimulation of cell proliferation, TNF and PGE_2 production by human bone cells in response to rhIL-1α was also examined. All of these studies demonstrated that the presence of the antibody exerted no detectable effects on any of the parameters measured. Furthermore, no effects of endogenous IL-6 were observed on the rhTNFα-induced cell proliferation or IL-1 production by human osteoblast-like cells. These studies do not support the notion that IL-6 has a role in the basal regulation of osteoblast cell activity or is responsible for mediating the actions of other osteotropic agents on human osteoblast-like cells.

INTRODUCTION

IL-6 has been shown to be produced by osteoblast-like cells from a variety of species such as rat (Feyen et al 1989; Lowik et al 1989), mouse (Ishimi et al 1990, Li et al 1991) and human (Littlewood et al 1991b, Linkhart et al 1991b). In human bone cells, IL-6 mRNA is constitutively expressed (Littlewood et al 1991b, Linkhart et al 1991b). Furthermore, quantities of bioactive basal endogenous IL-6 produced by human osteoblast-like cells range from 200 to 2050 pg/ml and 1950 to 6650 pg/ml in serum-free and 10% (v/v) FCS containing cell conditioned media respectively (Chapter 4). These quantities of IL-6 far exceed the levels of many other cytokines produced by human osteoblast-like cells such as TNF (Gowen et al 1990) and IL-1 (Hughes et al 1988). The functional significance of such high levels of IL-6 production by the human osteoblast-like cells is uncertain since addition of exogenous IL-6 appears to exert no effects on these cells. It is possible however, that endogenous levels of IL-6 may have a role either in the tonal regulation of osteoblast metabolism alone or may mediate the effects of other cytokines on a particular aspect of osteoblast function. These possibilities were examined by use of a sheep anti-human IL-6 antibody which, at a 1:1000 dilution, neutralized all endogenous IL-6 activity. This antibody was specific for IL-6 as determined in the B9 assay (see table 7.1). Effects of endogenous basal IL-6 on various stimulated and unstimulated osteoblast features were studied. These included cytokine production (IL-1 and TNF), PGE_2 production, proliferation and alkaline phosphatase and osteocalcin expression.

METHODS

Human osteoblast-like cells were plated out at a density of 5000 cells/well in a 96-well plate for alkaline phosphatase activity determinations and at 10000 cells/well in a 48-well plate for assessment of cell proliferation, osteocalcin, PGE_2, IL-1 and TNF production.

Cells were allowed to attach to the tissue culture plate for 24 h and then preincubated in medium containing 0.1% (w/v) BSA or 10% (v/v) FCS in the presence or absence of anti-IL-6 antibody (1:1000 final dilution) for 1-2 h prior to addition of appropriate test agents to induce the various parameters studied. The antibody was therefore present at 1:1000 dilution in half of the wells throughout the whole experiment which neutralized all endogenous IL-6 bioactivity in those wells. After an incubation period of 24-72 h, conditioned medium was removed and used for appropriate assay.

Assays for cell proliferation, PGE_2, osteocalcin, IL-1 and TNF production were all performed as described previously in Chapter 3 (methods).

IL-6 production was determined, by using the B9 assay (Chapter 2), in all wells in order to demonstrate that all bioactivity was neutralized in wells containing the antibody.

The procedure for the assessment of alkaline phosphatase activity was adapted for the 96-well plate micro-assay. Medium was removed from the wells and the cell layer washed twice with PBS. 4.64 mg of 4-nitrophenylphosphate was added to 10 ml of assay buffer (see Appendix 2) immediately prior to use (see Chapter 3 methods). 200 ul of this solution was then added to each sample and incubated at 37°C for 30-60 mins, or until optimal yellow/green colour developed. The absorbance at 410 nm was then measured on an ELISA platereader and results were expressed as a percentage of control absorbance value.

RESULTS

Stimulation of TNF production and cell proliferation induced by rhIL-1α (Fig 7.1)

Assessment of TNF production and cell proliferation were both studied in EMEM containing 10% (v/v) FCS since this was found to be the optimal condition to study these parameters. Under serum-free conditions, human osteoblast-like cells did not produce readily detectable levels of TNF using the WEHI cytotoxic assay (refer to Chapter 3). These experiments were conducted in the presence of indomethacin

(1.4 uM) as prostaglandins are known to inhibit cytokine release from various cell types (Kunkel and Chensue 1985). After a 72 h incubation, rhIL-1α (10^{-11}M) significantly enhanced cell proliferation and TNF production in human osteoblast-like cells. The stimulation of TNF synthesis and cell proliferation by rhIL-1α (10^{-11}M) was unaltered by the addition of the anti-IL-6 antibody (1:1000). There was also no difference between the levels of basal TNF production and cell proliferation in the presence or absence of anti-IL-6 antibody.

It was necessary to demonstrate that all bioactive IL-6 was neutralized in cultures containing antibody. A small amount of conditioned medium was retained from each experiment for this purpose. All IL-6 activity was consistently and completely neutralized when antibody was used at 1:1000 dilution.

Stimulation of PGE_2 production by rhIL-1α (Fig. 7.2).

rhIL-1α was an effective stimulator of PGE_2 production in human osteoblast-like cells. This was observed in serum-free culture after 72 h with as low a concentration as 10^{-13} M rhIL-1α although this effect was not always observed at such low concentrations. In this figure, the stimulatory effect was more marked in cultures without the presence of antibody. When cultures containing antibody were compared with the corresponding wells in the absence of antibody however, there were no significant differences. The stimulation of PGE_2 activity by 10^{-11} M rhIL-1α was very marked for human osteoblast-like cells +/- antibody. Complete neutralization of IL-6 bioactivity was observed upon addition of the IL-6 antibody.

Stimulation of cell proliferation by rhTNFα (fig. 7.3).

TNFα also stimulates proliferation of the human osteoblast-like cells, although it is less potent than IL-1 (Gowen 1988). This was reflected by a significant increase in cell proliferation first being detected at a concentration of 10^{-9} M rhTNFα after 72 h culture in medium containing 10% (v/v) FCS. The effect of TNFα on cell proliferation was most pronounced with the highest concentration of rhTNFα

(10^{-7} M) used. Presence of anti- IL-6 antibody did not affect TNFα-stimulated, or basal cell proliferation.

All IL-6 activity was neutralized in cultures containing the antibody.

Stimulation of IL-1 production by rhTNFα (fig. 7.4).

After 72 h culture in medium containing 10% (v/v) FCS, rhTNFα at a concentration of 10^{-8} M stimulated the basal IL-1 production although these levels were very low, amounting to 0.003 U/ml upon induction. The presence of endogenous IL-6 did not appear to affect either basal or TNF-stimulated production of IL-1. All endogenous IL-6 was blocked by the specific antibody.

Stimulation of alkaline phosphatase activity by 1,25$(OH)_2D_3$ (fig. 7.5).

In the absence of antibody, alkaline phosphatase levels were consistently induced by 1,25$(OH)_2D_3$ at 10^{-9} M and 10^{-8} M after 24 h culture in medium containing 10% (v/v) FCS. The presence of the antibody did not affect basal alkaline phosphatase expression, nor did it modulate the induction of enzyme activity by 1,25$(OH)_2D_3$. Under serum-free conditions, 1,25$(OH)_2D_3$ did not stimulate the basal alkaline phosphatase activity after a 24 h incubation period (data not shown). Wells containing antibody did not contain any endogenous IL-6 activity as assessed by the B9 assay.

Stimulation of osteocalcin synthesis by 1,25$(OH)_2D_3$ (fig. 7.6).

At a concentration of 10^{-8} M, 1,25$(OH)_2D_3$ consistently stimulated osteocalcin levels in human osteoblast-like cells after 72 h incubation in serum-free culture. The presence or absence of anti-IL-6 antibody did not alter either the basal or 1,25$(OH)_2D_3$-stimulated levels of this bone matrix protein. Furthermore, all IL-6 activity was neutralized in cultures containing antibody.

DISCUSSION

The present studies demonstrate that endogenous production of IL-6 does not affect any of the parameters studied either under basal or rhIL-1α, rhTNFα or 1,25(OH)$_2$D$_3$-stimulated conditions. Since ROS 17/2.8 cells are derived from a rat osteosarcoma cell line, the sheep anti-human IL-6 antibody was unable to cross react with rat IL-6 and therefore only studies with human trabecular bone cells could be investigated. The studies presented within this chapter would indicate that IL-6 does not influence several aspects of human osteoblast-like cell function although this series of experiments does not exclude the possible interactions of IL-6 with other factors or that other activities of human osteoblast-like cells are similarly unresponsive. The demonstration of the presence of IL-6 receptor mRNA (Chapter 6) in human osteoblast-like cells is an interesting observation especially since IL-6 seems to exert no effect on the trabecular bone cells. However, the possibility that mRNA levels are not converted to the final receptor protein can not be eliminated. Alternatively, although human osteoblast-like cells may contain a functional receptor, they may lack the gp 130 glycoprotein through which IL-6 transduces its signal (see Chapter 6 discussion). This aspect is currently being investigated. Another possibility is that the high endogenous levels of IL-6 produced by osteoblasts may be exerting a de-sensitising effect on the cell's functional receptor. The lack of effects of IL-6 on basal osteoblast function may therefore be due to a number of possibilities.

Furthermore, IL-6 did not modulate the effects of IL-1, TNF or 1,25(OH)$_2$D$_3$ with respect to the various osteoblast features studied (discussed in chapter 3). The theory that IL-6 may be a secondary agent by which IL-1 can mediate its effects arise from a number of studies: IL-1, TNF and IL-6 are produced by a wide range of cells, and IL-1 and TNF further induce IL-6 production in a number of these cell types including fibroblasts (Van Damme et al 1987a, 1987c, Elias and Lentz 1990), monocytes (Aarden et al 1987) and osteoblasts (Ishimi et al 1990, Littlewood et al 1991b, Linkhart et al 1991b). As discussed in previous chapters, these cytokines

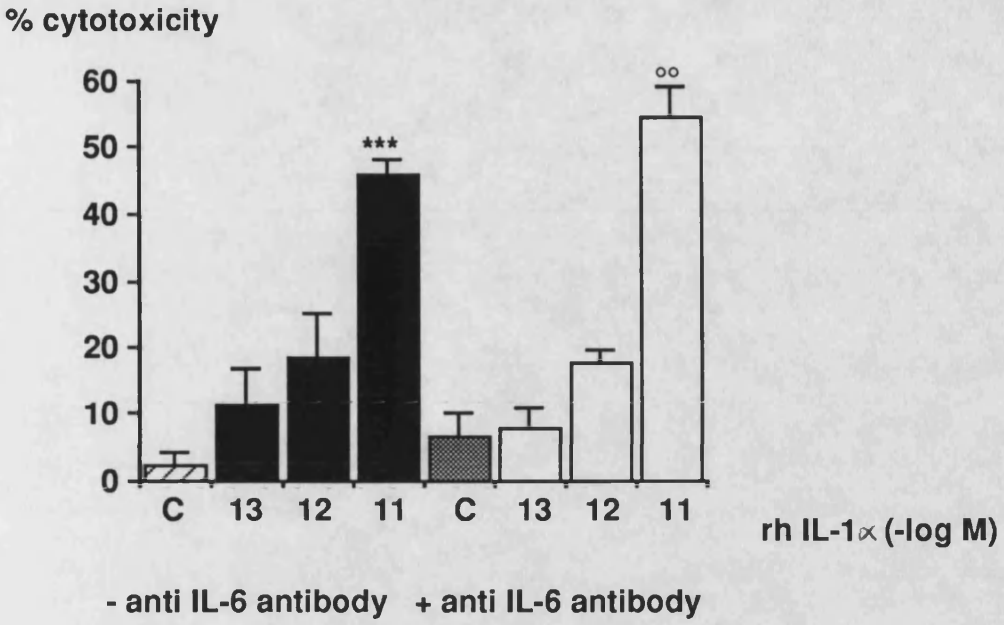
have many pleiotropic actions, many of which overlap, including the induction of the acute phase response and proliferation of thymocytes. It is possible that IL-1 and TNF may be exerting their effects in part at least, via the production of a secondary cytokine such as IL-6. Therefore, as well as examining the effects of endogenous IL-6 on osteoblast function, this chapter was designed to investigate whether the various effects of IL-1 and TNF were mediated via a secondary cytokine such as IL-6. Others have observed that IL-6 is able to mediate the IL-1 response in a number of different environments, although some of the evidence presented is circumstantial rather than direct. Guerne et al (1989) observed that IL-1 and TNF are important in mediating inflammatory joint destruction through their ability to stimulate production of proteases and prostaglandins by synoviocytes. Guerne and co workers (1989) in the same report also demonstrated that IL-1 and TNF induce IL-6 expression by synoviocytes; hence it is possible that IL-1 and TNF are causing joint destruction via IL-6 production. Antibody studies are required to address this point. Nietfeld et al (1990) using human cartilage demonstrated that IL-1 stimulated IL-6 production and furthermore IL-6 was partially responsible for the IL-1 induced inhibition of proteoglycan synthesis. By using anti-IL-6 antibody, this group was able to demonstrate that IL-1 was exerting its effects partially by induction of IL-6. Black et al (1990, 1991b), using neonatal mouse calvariae, observed that anti-IL-6 antibody inhibited resorption due to IL-1 and TNF. This latter work is controversial however, as other groups have been unable to demonstrate any effects of IL-6 on resorption (Barton and Meyer 1990, Al-Humidan et al 1991). Furthermore, using anti-IL-6 antibody, Al-Humidan et al (1991) demonstrated that IL-6 exerted no effect on IL-1 or TNF- stimulated resorption in the same system. Bataille et al (1991) observed that during the early stages of multiple myeloma, there was an increase in resorption accompanied by increased osteoblast numbers and generation of new osteoclasts. They demonstrated the presence of several cytokines in the myeloma milieu including IL-1, TNF and IL-6, which they proposed acted in cascade and synergy to enhance

the pathogenesis of the disease. Obviously antibody studies are required in order to investigate which cytokines are mediating the effects of the other factors in the milieu.

Hence, although IL-6 is capable of mediating the response of cytokines such as IL-1 and TNF in various cells within the bone microenvironment, from the studies presented in this chapter, this does not seem to be the case for the human osteoblast.

Fig. 7.1 Effect of endogenous IL-6 production on rhIL-1 -stimulated TNF production and proliferation in human osteoblast-like cells.

Human osteoblast-like cells were incubated with rhIL-1 in the presence or absence of sheep anti-human IL-6 antibody for 72 h in 10% (v/v) FCS-containing EMEM. The cell layer was subsequently assayed for proliferation (middle graph) and the conditioned medium was removed for TNF (top graph) and IL-6 bioassay. Mean ± S.E.M. Significant difference from control (- antibody) *p<0.05, **p<0.01, ***p<0.001. Significant difference from control (+ antibody) °°p<0.01. There was no significant difference between wells ± antibody for any of the above parameters investigated. Neutralization of IL-6 activity in the cultures containing the sheep anti-human IL-6 antibody was determined by the B9 assay (bottom graph). Representative experiment (n=3).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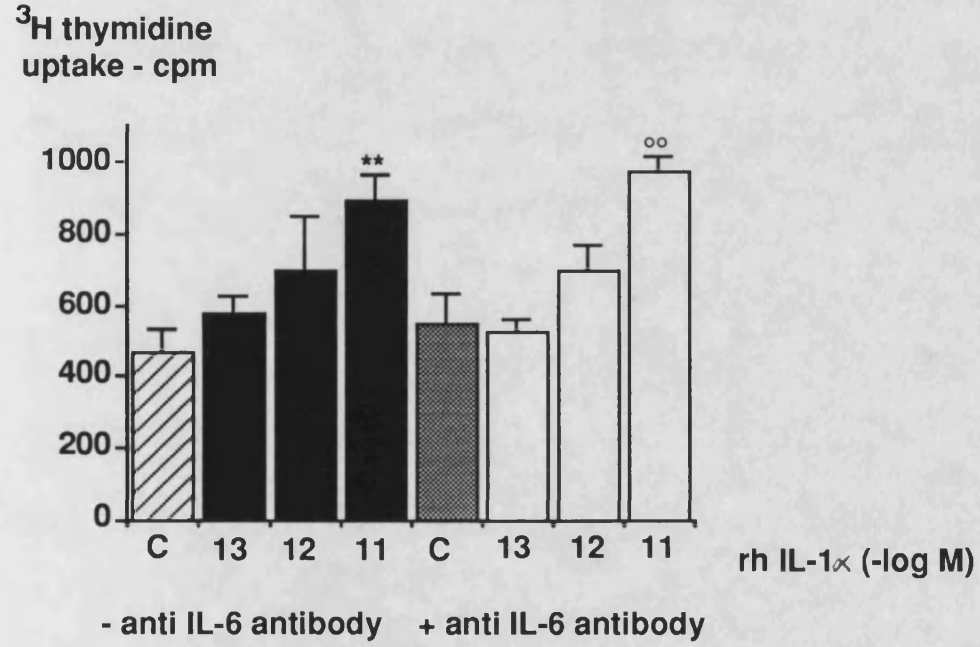
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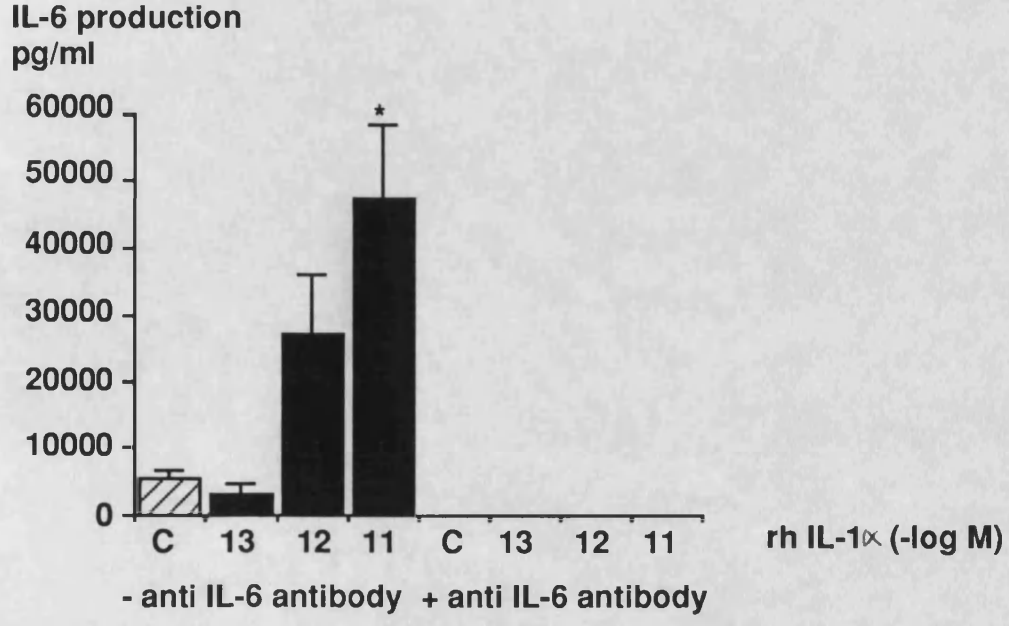
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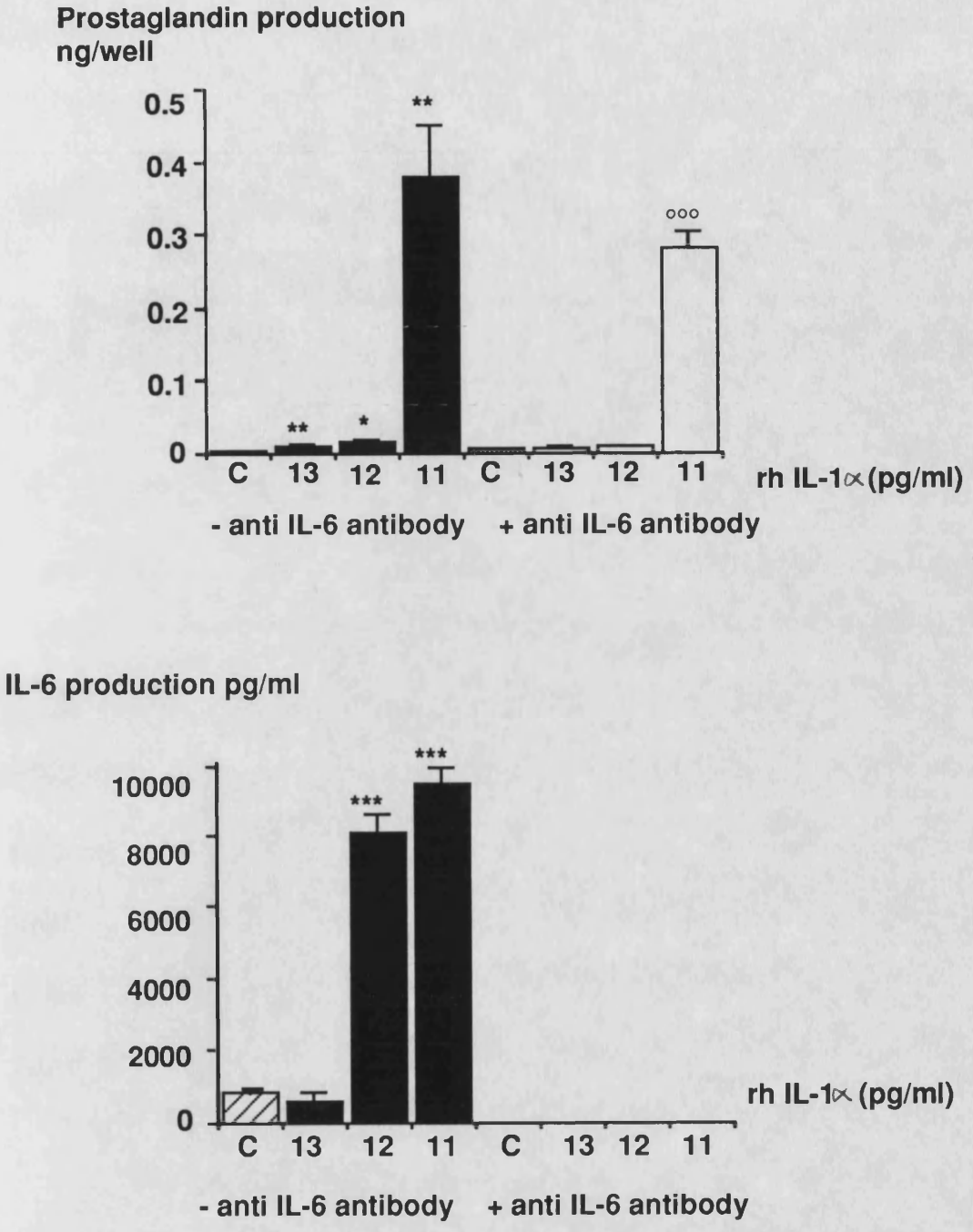


Fig. 7.2 Effect of endogenous IL-6 on rhIL-1∝ stimulated prostaglandin E_2 levels in human osteoblast-like cells.

Human trabecular bone cells were incubated with rhIL-1a in the presence and absence of antibody for 72 h in 0.1% BSA-containing EMEM . The bone cell conditioned medium was removed and assayed for PGE_2 levels (top graph) and IL-6 bioactivity. Mean ± S.E.M. Significant difference from control (- antibody) *p<0.05, **p<0.01, ***p<0.001. Significant difference from control (+ antibody) °°°p<0.001. There was no significant difference between wells ± antibody.
Neutralization of IL-6 activity in the cultures containing the antibody was determined by the B9 assay (bottom graph). Representative experiment (n=3).

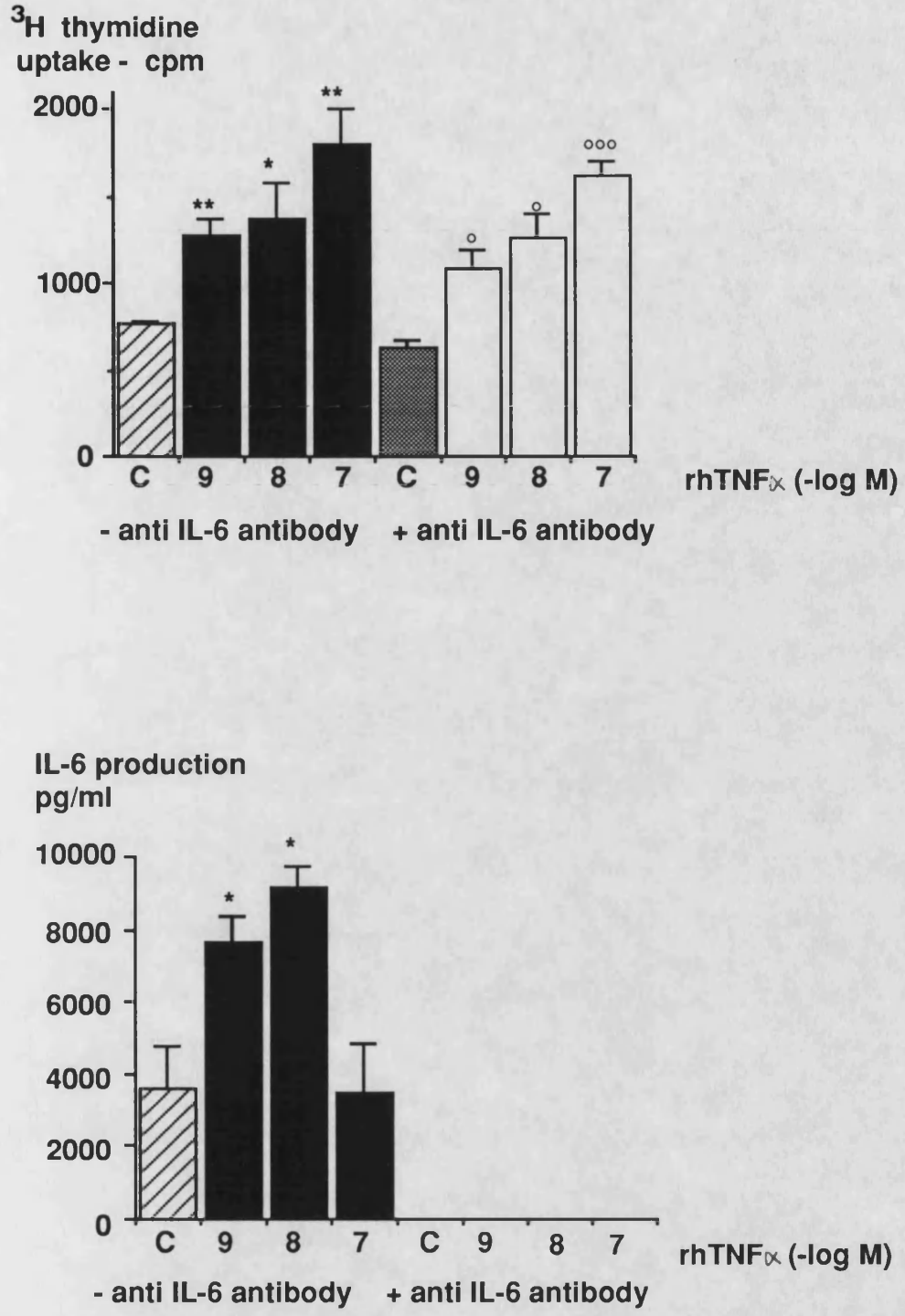


Fig. 7.3 Effect of endogenous IL-6 on rhTNFα-stimulated proliferation in human osteoblast-like cells.

Human trabecular bone cells were incubated with rhTNFα in the presence or absence of sheep anti-human IL-6 antibody for 72 h in 10% (v/v) FCS- containing EMEM. The cell layer was assessed for proliferation (top graph) and the conditioned medium was stored for IL-6 bioassay. Mean ± S.E.M. Significant difference from control (- antibody) *p<0.05, **p<0.01. Significant difference from control (+ antibody) °p<0.05,°°°p<0.001. There was no significant difference to wells ± antibody.
Neutralization of IL-6 activity in the cultures containing the antibody was determined by the B9 assay (bottom graph). Representative experiment (n=3).

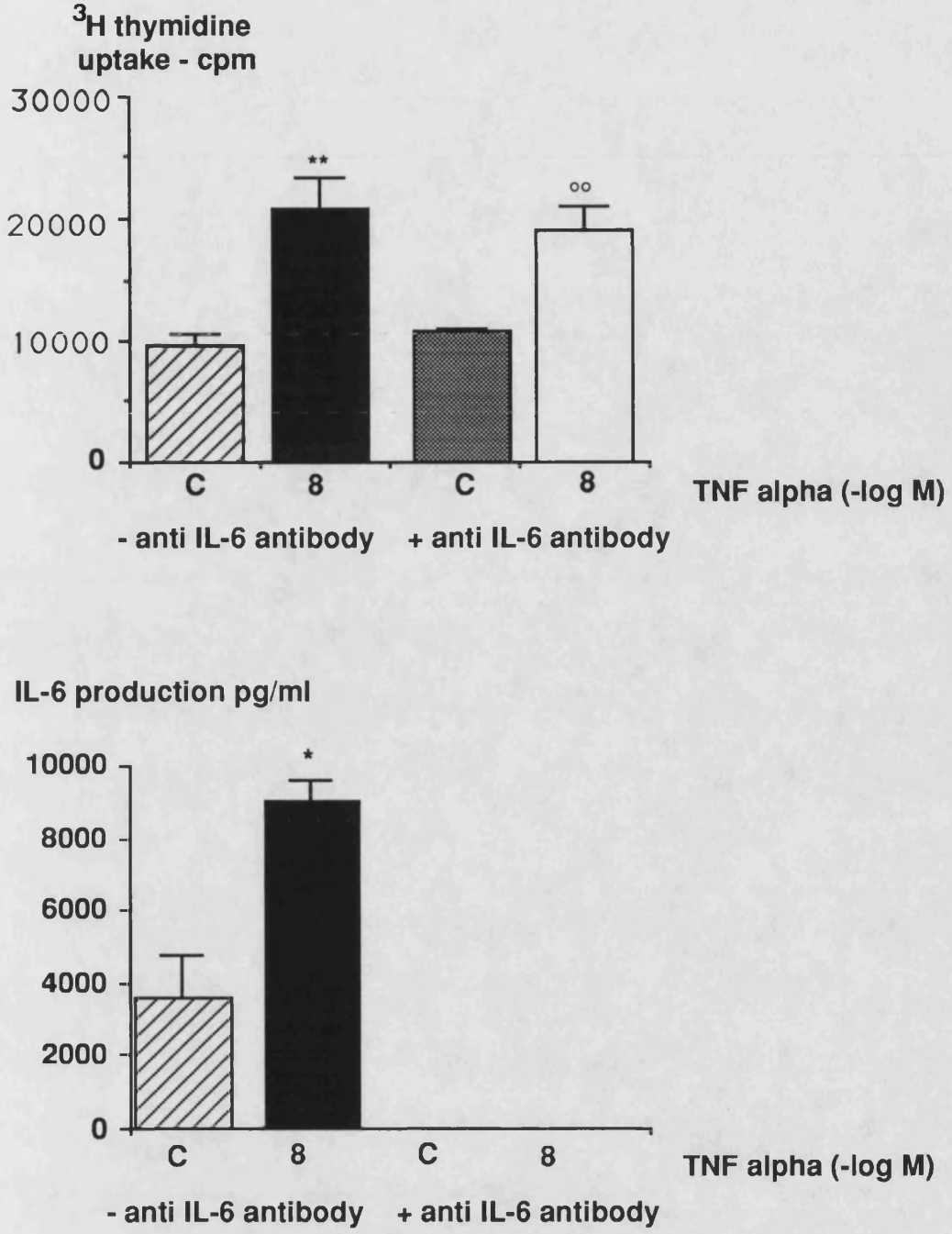


Fig. 7.4 Effect of endogenous IL-6 on rhTNFα- stimulated IL-1 levels in human osteoblast-like cells.

Human osteoblast-like cells were treated with rhTNFα in the presence and absence of sheep anti-human IL-6 antibody in 10% (v/v) FCS-containing EMEM for 72 h culture. The bone cell conditioned medium was removed and assayed for IL-1 and IL-6 bioactivity. Mean ± S.E.M. Significant difference from control (- antibody) *$p<0.05$, **$p<0.01$. Significant difference from control (+ antibody) °°$p<0.01$. There was no significant difference between wells ± antibody.

Neutralization of IL-6 activity in the cultures containing the antibody was determined by the B9 assay (bottom graph). Representative experiment (n=3).

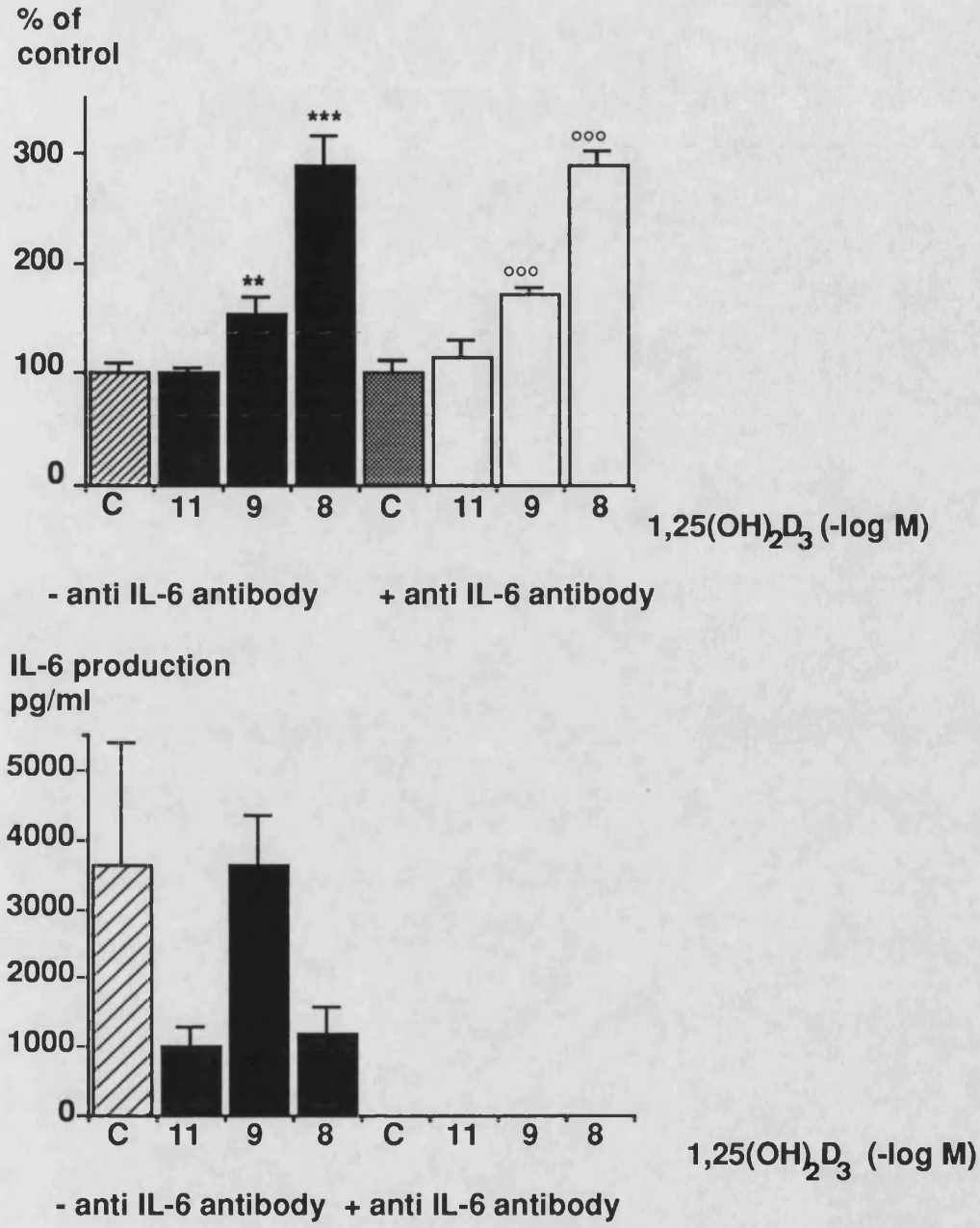


Fig. 7.5 The effect of endogenous IL-6 on $1,25(OH)_2D_3$-stimulated alkaline phosphatase levels in human osteoblast-like cells.

Human osteoblasts were incubated with $1,25(OH)_2D_3$ in the presence and absence of sheep anti-human IL-6 antibody for 24 h in 10% (v/v) FCS-containing EMEM. Alkaline phosphatase activity in the cell layer was measured on a spectrophotometer at 410 nm (top graph). Mean ± SEM. Significant difference from control (- antibody) where absorbance at 410 nm was 0.139, $^{**}p<0.01$, $^{***}p<0.001$. Significant difference from control (+ antibody) where absorbance at 410 nm was 0.142 $^{°°°}p<0.001$. There was no significant difference between the wells treated with antibody and wells without antibody. Neutralization of IL-6 activity in the cultures containing the antibody was determined by the B9 assay (bottom graph). Representative experiment (n=3).

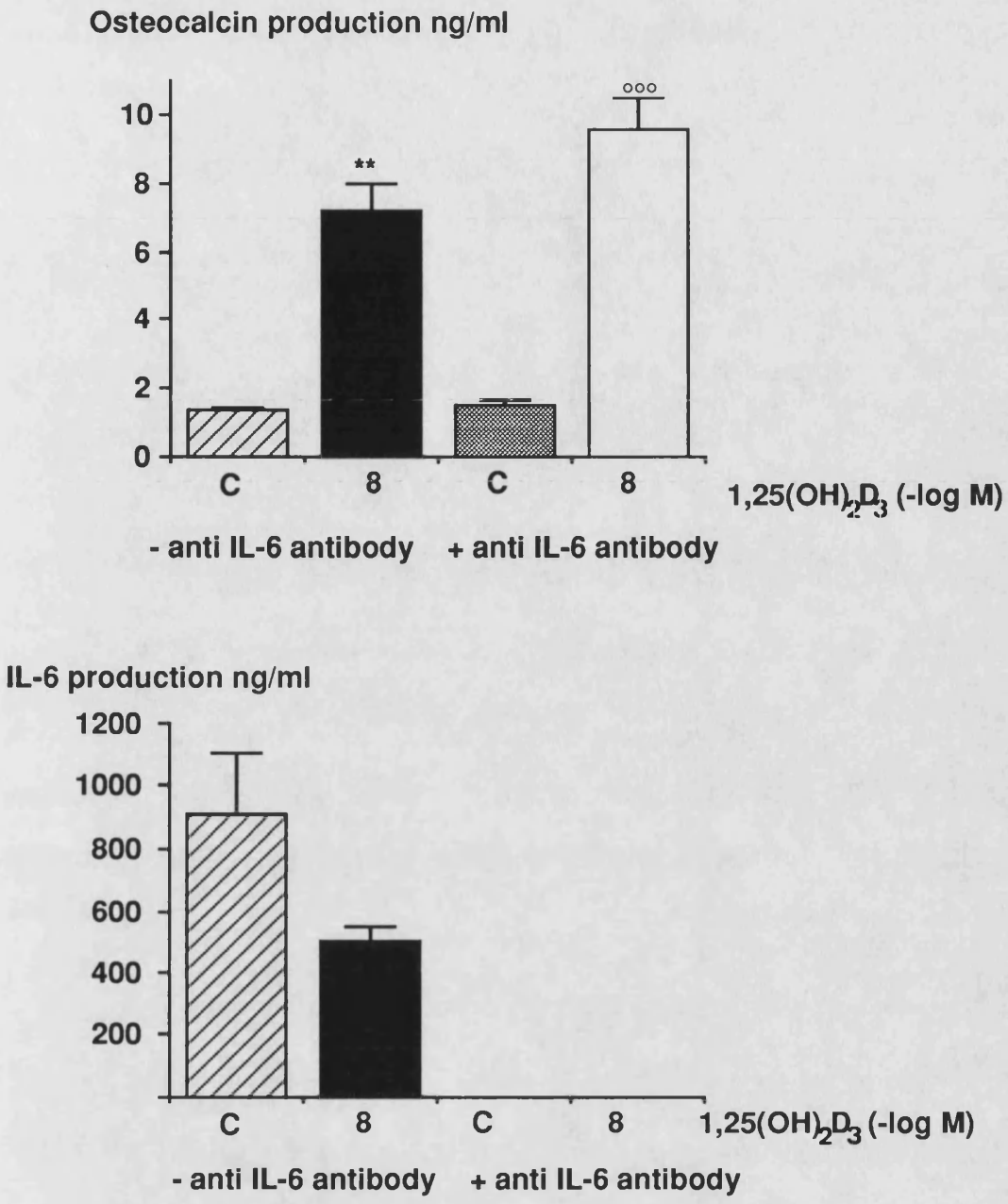


Fig. 7.6 Effect of endogenous IL-6 on $1,25(OH)_2D_3$ -stimulated osteocalcin levels in human osteoblast-like cells.

Human trabecular bone cells were cultured with $1,25(OH)_2D_3$ in the presence or absence of sheep anti-human IL-6 antibody in 0.1% BSA-containing EMEM for 72 h culture. The conditioned medium was removed and assessed for osteocalcin content (top graph) and IL-6 bioactivity. Mean ± S.E.M. Significant difference from control (- antibody) $^{**}p<0.01$. Significant difference from control (+ antibody) $^{°°°}p<0.001$. There was no difference between wells ± antibody. Neutralization of IL-6 activity in the cultures containing the antibody was determined by the B9 assay (bottom graph). Representative experiment (n=3).

GENERAL DISCUSSION

From the data presented within this thesis, it can be seen that IL-6 does not affect osteoblast function with respect to proliferation, differentiated phenotype, and production of cytokines and prostaglandins. This could be due to a number of factors such as

a) inappropriate osteoblast parameters studied:-

Others have observed a slight depression in collagen type I production by murine osteoblasts (Ishimi et al 1990, Fang and Hahn 1991). Whether this is the case for the human osteoblasts remains to be elucidated. In addition, further information may be gained by the investigation of whether IL-6 modulates the expression of integrins and production of bone matrix proteins such as osteopontin. This study could provide information about whether the osteoblasts are induced to interact with the matrix and attach to the bone surface. Several bone matrix proteins such as thrombospondin (Gehron-Robey et al 1989) and osteopontin (Oldberg et al 1986) contain an RGD sequence which is associated with cell attachment and spreading. Furthermore, integrin expression by cells also promotes attachment by binding to recognised sequences. The RGD sequence is a ligand for some of these integrins for example (Dedhar 1990, Ruoslahti and Yamaguchi 1991). Human osteoblasts express the $\alpha 1$ integrin, the expression of which is upregulated by the addition of IL-1 (J.Clover, personal communication). As this integrin subunit is capable of association with collagen (Dedhar 1990), it supports the theory that osteoblasts are capable of attachment to the bone surface. In addition, Dedhar (1989) reported that the $\beta 1$ integrin subunit was induced in human osteosarcoma lines after exposure to IL-1. Whether IL-6 mimicks the IL-1 action in human osteoblasts remains to be studied. Certainly other effects of IL-1 on various human osteoblast features such as proliferation and prostaglandin and cytokine production, were not shared by IL-6.

b) osteoblasts *in vitro* may not behave in the same way as ones *in vivo*: -

Little is known about the behaviour of cells *in vivo*. *In vitro* study is less complicated and can be valuable for demonstration of general trends. An example of this is evident by the fact that IL-1 induces proliferation of osteoblasts both *in vitro* (Gowen 1988) and *in vivo* (Boyce et al 1989). *In vitro* study does have its limitations however, in that generally the system is over simplified and often effects of a single agent on an isolated cell type are studied. Therefore, possibility of interaction with other agents, cell types and matrix is not explored. It is possible that IL-6 may have effects on osteoblasts in combination with other factors produced by some other cell type absent in tissue culture. Alternatively, IL-6 may act on another cell type resulting in the release of a factor that may affect the osteoblasts. Another problem is encountered by using various clonal osteoblast cell lines as a potential model system. They all behave differently in culture which can serve to confuse the issue rather than clarify it. The rat osteosarcoma line, ROS 17/2.8, exhibits a very different spectrum of actvities to the human trabecular bone cells *in vitro* (Chapter 3). The former cells do not produce bioassayable amounts of TNF, exhibit lower prostaglandin and IL-6 levels and higher alkaline phosphatase levels. Furthermore, their response to osteotropic agents differ, for example PTH stimulates IL-6 production in the rat osteosarcoma line, but not in the human osteoblast-like cells. Therefore while these systems are useful for the study of osteoblast functions, results must be interpreted with some caution.

Culture on plastic rather than on the bone surface or matrix components also has an influence on osteoblast function and cytokine production. For example, adherence of macrophages and monocytes to plastic can produce a powerful signal for the production of TNFα and IL-1. This apsect, amongst others is reviewed by Nathan and Sporn 1991. Culture of osteoblasts on collagen gels reduces 1,25$(OH)_2D_3$- induced alkaline phosphatase and osteocalcin expression (J. Clover, personal communication). Hence it is likely that matrix components have a bearing on remodelling functions (Nathan and Sporn 1991). They suggested that cytokines are intercellular signal proteins that coordinate remodelling of tissues in association with the extracellular matrix. There are many ways that the two interact, such as adherence to matrix could induce cells to

produce cytokines, or cytokines themselves might affect cell adhesion receptors (integrins - as discussed earlier). Combined requirement for cytokine and matrix signals may be important for localising responses of cells to a cytokine (such as IL-6) that is widely distributed. Cytokine interactions on cells *in vitro* need to take into account the extracellular milieu of the responding cells in vivo. Nathan and Sporn (1991) postulated that *in vitro* tissue culture on isolated cell populations often leads to contradictory effects for a particular agent and therefore is a relatively unsatisfactory technique.

Future work should take this into consideration, and culture of cells within 3 dimensional matrices containing components of the bone matrix may provide us with more meaningful results.

In situ hybridisation and immunolocalisation, although difficult to quantitate, have the potential to provide valuable information about which cells are expressing particular proteins at a certain time. Also information on whether expression alters under different environmental conditions is relatively easily obtained with these techniques. In Chapter 4, it was demonstrated that human osteoblasts produced high constitutive levels of IL-6 mRNA *in vitro* which was in contrast to the fluctuating levels of IL-6 mRNA expression observed in tissue sections by *in situ* hybridisation. Therefore, it would appear as though the synthesis of IL-6 *in vitro* does not accurately reflect expression *in situ*. Co-localisation of IL-6 mRNA with the expression of various markers of osteoblast differentiation has the potential to provide valuable information as to whether the IL-6 gene is regulated at different stages of osteoblast development and this may give an indication of the role of IL-6.

c) osteoblasts do not have functional receptors:-

In Chapter 6, mRNA for the IL-6R was constitutively produced by human osteoblast like cells. It is possible that the mRNA is not converted to the final mature protein or alternatively, the receptor is expressed but associated signalling components such as gp 130 (Taga et al 1989) are absent. Another possibility is that the osteoblasts do not contain IL-6-inducible nuclear factors which bind to responsive elements of certain

genes to alter their transcription as visualised in hepatocytes (Poli et al 1990). These possibilities need to be explored by the use of immunoprecipitation techniques, and by Northern analysis as cDNA probes for gp 130 (Hibi et al 1990) and nuclear factor IL-6DBP (Poli et al 1990) have been isolated.

d) endogenous levels of IL-6 mask exogenous IL-6:-

In Chapters 4 and 5, it was demonstrated that human osteoblasts produce large quantities of IL-6 (B9 bioassay, Northern analysis, immunolocalisation and *in situ* hybridisation). Therefore another explanation for the lack of effects of IL-6 on human osteoblasts may be that high endogenous IL-6 levels mask the exogenously added cytokine. This possibility was eliminated by use of a monoclonal antibody to IL-6. This neutralised all IL-6 endogenous activity in culture (assessed by the B9 assay). The removal of IL-6 did not modulate various basal or stimulated osteoblast parameters such as proliferation, cytokine and prostaglandin production, and differentiated phenotype such as alkaline phosphatase and osteocalcin expression. Therefore, IL-6 was not responsible for the modulation of the above parameters in human osteoblasts. However, this does not exclude the possibility that endogenous IL-6 levels are modulating other functions of osteoblasts not studied in this thesis.

e) The half life of IL-6 *in vivo* and *in vitro* is another factor to take into consideration and presence of inhibitors or protective carrier proteins will affect this.

α2 macroglobulin is known to associate with IL-6 to form a soluble complex which is transported in the circulation (Matsuda et al 1989). This provides a mechanism whereby IL-6 is protected from degradation and is transported systemically without inhibiting its function. Novick et al (1989) demonstrated the presence of the extracellular portion of the IL-6R in human urine, which was capable of binding IL-6 but did not inhibit IL-6 activity. Saito et al (1991) developed an ELISA in the murine system for the detection of the truncated IL-6 receptor whereas the equivalent ELISA in the human requires development. Presence of α2 macroglobulin and soluble IL-6R would provide valuable information as to whether the conditions *in vivo* and *in vitro* are different, resulting in alterations in the lifespan of IL-6. It is of interest to note that a soluble form

of the TNF receptor exists in the circulation which inhibits TNF activity (Nophar et al 1990). Whether a specific inhibitor for IL-6 exists remains to be elucidated.

The study presented in Chapter 5 has also shown that osteoclasts produce IL-6 mRNA. Evidence would suggest that they may produce the IL-6 protein as this was visualised by immunolocalisation on isolated osteoclasts derived from osteoclastoma tumour. Immunolocalisation studies need to be repeated in tissue sections using a more appropriate negative control as the non immune sheep serum used in these studies may have contributed to the high non specific background seen in tissue sections.
From evidence presented in this thesis and from other studies (Ohsaki et al 1991, Qi et al 1991, Merry et al 1992b, Hoyland et al 1992) it would appear that osteoclasts are capable of synthesizing several cytokines and therefore do not exert a purely resorptive role. By expressing mRNA for various cytokines, the osteoclasts may signal to other cells within the bone microenvironment. For example, it is possible that osteoclasts are producing IL-6 in order to maintain hematopoiesis resulting in the promotion of the maturation of immature osteoclasts (discussed later). The possible production of cytokines such as TNF by the osteoclast (Qi et al 1991) is likely to affect the proliferation of the osteoblasts. Gowen (1988) demonstrated that *in vitro*, TNF increased osteoblast proliferation and down-regulated the differentiated phenotype of osteoblasts which may serve to increase the available osteoblast pool. As osteoblasts are capable of producing TNF themselves (Gowen et al 1990, Merry et al 1990), it appears that this cytokine can act in an autocrine as well as a paracrine fashion. It therefore seems likely that osteoclasts are capable of modulating osteoblast function in a number of ways. Merry et al (1991) was unable to demonstrate the production of TGFβ in osteoclasts by *in situ* hybridisation. However, from the work of Oreffo et al (1989) and Pfeilschifter et al (1990a 1990b), it is likely that osteoclasts activate the latent TGFβ complex present in the matrix, which serves as a chemoattractant for osteoblasts. Other growth factors and bone matrix proteins will be released during the

process of resorption and these will also have influences on the osteoblasts as well as on the other cells within the vicinity of the resorbing osteoclasts.

Further *in situ* studies are required to demonstrate the repertoire of factors produced by osteoclasts and this will contribute to the understanding of the role of this cell in remodelling. In addition, since isolation of osteoclasts is a difficult procedure and their phenotype does not appear to be stable in culture, *in situ* hybridisation is a useful tool for studying the osteoclast. There are limitations to *in situ* hybridisation, as handling of the tissue is critical and sections need to be demineralised, resuting in the loss of cells and available mRNA. However, *in situ* hybridisation is particularly valuable for the study of the behaviour of cells and is more likely to represent an *in vivo* situation than tissue culture conditions. Whilst tissue culture is useful for the simplified study of a particular factor on an isolated cell type, the limitations are numerous as discussed earlier. More involved studies are now required to study interactions of cells with factors within their local milieu.

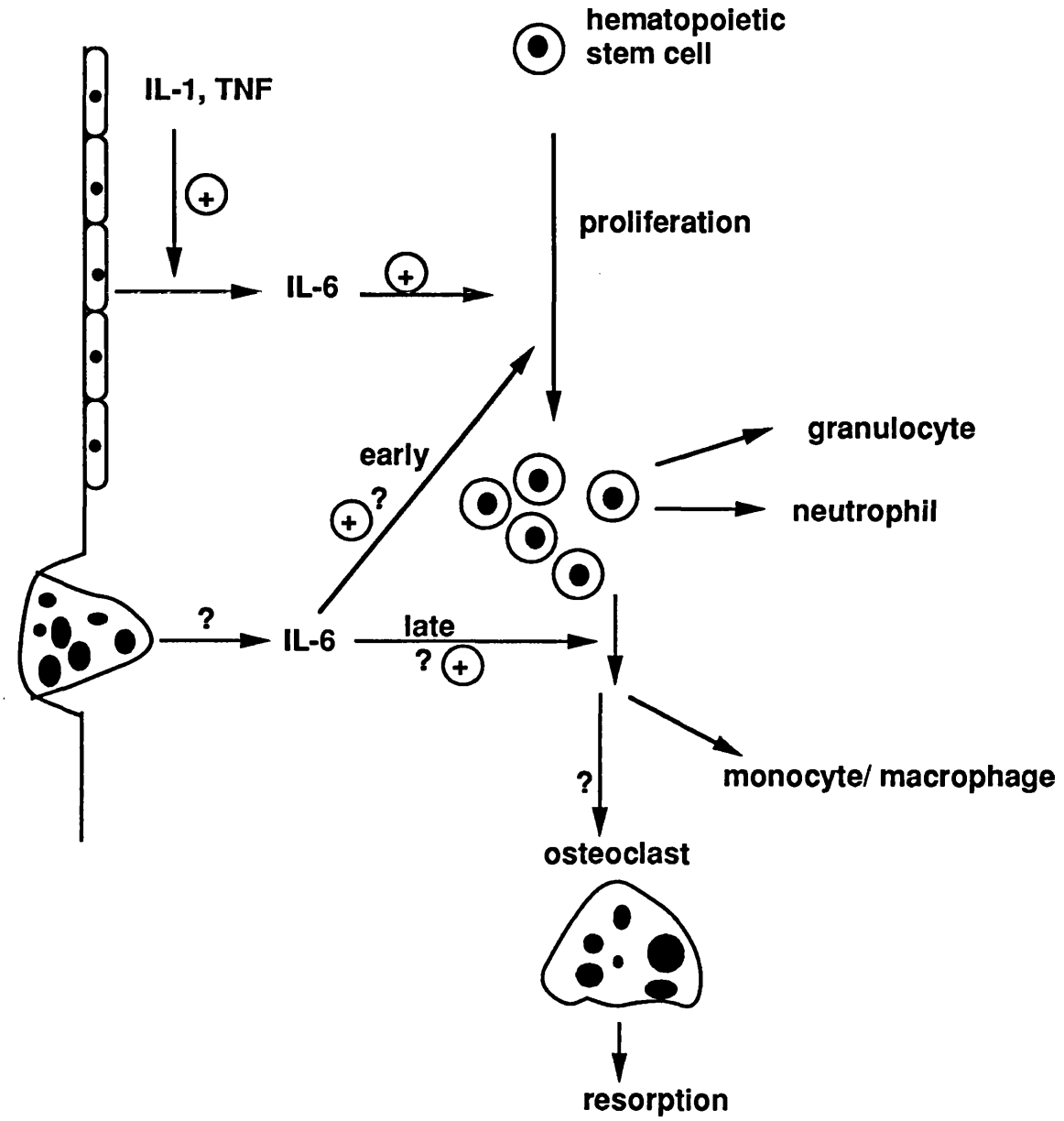
From the studies presented within this thesis, it appears that IL-6 is produced by osteoblasts and exerts a paracrine role in that it acts on other cells within the tissue. The fluctuating levels of this cytokine produced by different populations of osteoblasts seen in chapter 5, would indicate that IL-6 expression during remodelling is not at a constant level. Furthermore, IL-1 and TNF-stimulated IL-6 mRNA levels were transiently increased in human osteoblasts (Chapter 4) which would support the theory that IL-6 levels within the bone microenvironment are variable. It is possible that IL-6 concentrations need to reach a threshold level before its actions become effective *in vivo*. More detailed *in situ* studies are required to establish whether these ideas have foundation.

Whether IL-6 exerts paracrine or autocrine effects on osteoclasts remains to be elucidated. The study of the presence of the IL-6 receptor on osteoclasts by *in situ* and immunolocalisation techniques would be useful in this respect. There are several conflicting studies concerning the role of IL-6 on bone resorption (Barton and Mayer

1990, Al-Humidan et al 1991, Black et al 1991b, Ishimi et al 1990, Lowik et al 1989). Furthermore, IL-6 is increased by the resorptive agents IL-1 and TNF and may therefore serve to prolong resorption (Black et al 1990) or inhibit resorption (Al-Humidan et al 1991) by these factors.

The majority of evidence suggests that IL-6 acts at some stage on the immature osteoclast precursors to promote osteoclastogenesis. There are several lines of evidence to support this theory. Many groups have observed that IL-6 promotes maturation of hematopoietic stem cells along the granulocyte/macrophage lineage (Ikebuchi et al 1987, Hoang et al 1988, Wong et al 1988, Suda et al 1988, Gardner et al 1990). Kurihara et al (1990) demonstrated the induction of osteoclasts by IL-6 in long-term human marrow culture and Lowik et al (1989) observed that IL-6 only promoted resorption in murine bones containing osteoclast precursors. This suggests that IL-6 exerts a permissive role on the promotion of resorption, in that IL-6 increases production of osteoclast precursors rather than acting on the mature phenotype.

The schematic diagram shows the proposed role of IL-6 involvement in bone (Fig. 8.1).


hematopoietic
stem cell
IL-1, TNF
proliferation
IL-6
granulocyte
early
?
neutrophil
?
IL-6
late
?
monocyte/ macrophage
?
osteoclast
resorption

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